Spotlight on vitamin D receptor, lipid metabolism and mitochondria: Some preliminary emerging issues

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Running title:

Mitochondrial effects of vit.D on lipid metabolism

Abstract

Transcriptional control and modulation of calcium fluxes underpin the differentiating properties of vitamin D ($1,25(OH)_2D_3$). In the latest years however few studies have pointed out the relevance of the mitochondrial effects of the hormone. It is now time to focus on the metabolic results of vitamin D receptor (VDR) action in mitochondria, which can explain the pleiotropic effects of $1,25(OH)_2D_3$ and may elucidate few contrasting aspects of its activity. The perturbation of lipid metabolism described in VDR knockout mice and vitamin D deficient animals can be revisited based on the newly identified mechanism of action of $1,25(OH)_2D_3$ in mitochondria. From the same point of view, the controversial role of $1,25(OH)_2D_3$ in adipogenesis can be better interpreted.

Keywords: $1,25(OH)_2D_3$; vitamin D receptor; mitochondrial metabolism; lipid metabolism; vitamin D/VDR deficiency; adipogenesis.

Abbreviations: VDR, vitamin D receptor; RXR, retinoid X receptor; VDR KO, VDR knockout mice; WT, wild type; WAT, white adipose tissue; BAT, Brown adipose tissue; UCP, uncoupling protein; THR, thyroid hormone receptor; PPARγ, peroxisome proliferator-activated receptor gamma; OCR, oxygen consumption rate; CPT2: carnitine palmitoyltransferase 2; LXR, liver X receptor; DRIP 205/MED1, vitamin D-interacting protein 205/mediator complex subunit 1; TCA cycle, tricarboxylic acid cycle; C/EBPβ, CCAAT/enhancer binding protein beta.

1. Introduction

Many biological functions of the active form of vitamin D $1,25(OH)_2D_3$ are mediated by the control exerted by its receptor VDR on nuclear transcription. VDR acts as a transcription factor, migrating into nuclear compartment upon ligand binding and together with its binding partner retinoid X receptor alpha (RXRα) it recruits coactivators or corepressors to enhance or repress the transcription of many genes. Although $1,25(OH)_2D_3$ nuclear activity has generally been described as a promoter of differentiation, often the effects exerted by $1,25(OH)_2D_3$ are not detectable or even opposite [1-2]. A defective nuclear function of VDR has been described as the altered switch between coactivators and corepressors associated to VDR [3,4], but alone this perturbation does not account for the contrasting effects triggered by $1,25(OH)_2D_3$; moreover the non-genomic activity of VDR, mainly mediated by calcium fluxes, is responsible for additional effects that may change the response to $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$ controls calcium homeostasis through genomic and non genomic pathways. As transcriptional factor, vitamin D receptor (VDR) upregulates calcium transporters; for example it induces the expression of the ion channel transient receptor potential vanilloid type 6 (*TRPV6,* the main mediator of calcium transport across the plasmamembrane of intestinal epithelium [5]), and it increases the messenger of the calcium sensing receptor CaR [6], sensitizing the keratinocytes to extracellular calcium and calcium-triggered differentiation.

The main non genomic action of $1,25(OH)_2D_3$ consists in the regulation of calcium fluxes by direct interaction with plasma membrane proteins. In fact VDR localizes to the caveolar domains of plasma membrane, like other members of the steroid receptor superfamily, and promotes rapid membrane-initiated responses (reviewed in [7,8]). The outcomes of the membrane bound VDR activity influence nuclear transcription; for example the opening of voltage-gated calcium channels [9-12] or the generation of second messengers (as summarized in [13]) contributes to a concerted genomic and non-genomic regulation of many vitamin D-responsive genes. The aim of this review is to widen the analysis of the complex cross-talk between nuclear and extranuclear effects of

$1,25(OH)_2D_3$ mediated by different VDR pools; for this purpose, we discuss the mitochondrial effects of $1,25(OH)_2D_3$ and their metabolic consequences. For further details on the activity of $1,25(OH)_2D_3$, the reader is referred to very comprehensive reviews on vitamin D metabolism [14,15], VDR transcriptional activity [16,17] and regulation [18].

2. VDR: a nuclear receptor with a penchant for mitochondria

In addition to the nuclear effects of $1,25(OH)_2D_3$, recently a new mechanism of action has been suggested by the described localisation of VDR in mitochondria of platelets, megakaryocytes [19] and keratinocytes [20], where the translocation is mediated by the permeability transition pore activity [20]. Indeed, the mitochondrial function of VDR has been elucidated by subsequent works on cancer cell lines [21], keratinocytes [22] and brown adipocytes [23] that have demonstrated that $1,25(OH)_2D_3$ suppresses mitochondrial respiration, hence VDR has been defined a gatekeeper of mitochondrial respiratory chain activity. The consequences of the negative modulation of respiratory chain exerted by VDR are important for cellular bioenergetics as well as for biosynthetic pathways; in fact the mitochondrial activity of $1,25(OH)_2D_3$ supports both cell growth and differentiation and this newly discovered metabolic effect relies on the switch from oxidative to biosynthetic metabolism [21,22]. Given its dual nuclear and mitochondrial activity, it is tempting to speculate that $1,25(OH)_2D_3$ has a central role both in the regulation of gene sets and in supporting the metabolic requests evoked by nuclear signaling. In fact it is reasonable to think that the mitochondrial activity is modulated in order to adapt cellular metabolism to the nuclear differentiation program or proliferative nuclear signaling.

The metabolism that most obviously is affected by the mitochondrial control is the lipidic one; in fact both the catabolic pathways and the formation of precursors for biosynthetic purposes take place in mitochondria. In this article, we provide an overview of the link between the activity of $1,25(OH)_2D_3$ and lipid metabolism, and we put forward new and speculative hypotheses on the effects of VDR signaling in mitochondria, by suggesting that the combined nuclear and

mitochondrial action of $1,25(OH)_2D_3$ might support the metabolism of the adipose tissue and skin as well as many others.

3. The lessons learned from VDR knockout mice

Due to the central role of $1,25(OH)_2D_3$ in calcium homeostasis and deposition in bones, the silencing of VDR in knockout (KO) mice causes a defective calcium absorption and skeletal remodeling. Three independent models of VDR null mice were generated by disruption of the DNA binding domain of VDR, ablating either the first or the second zinc finger (exon 2 and 3, respectively) (Tokyo [24], Boston [25], Leuven [26] models); a fourth group (Munchen model [27]) produced a mouse that expressed a VDR protein with a deletion of the first zinc finger, thus unable to bind DNA, but with normal ligand binding affinity. All the models developed hypocalcemia, hypophosphatemia. secondary hyperparathyroidism, severe rickets and osteomalacia [24-27] due to the lack of functional VDR. Also the non-genomic effects of VDR were lost [27]. Besides the expected hypocalcemic and bone defective phenotype, VDR knockout mice are a good model to study the impact of VDR-mediated $1,25(OH)_2D_3$ activity in extra-skeletal tissues and general metabolism in vivo, including the role of $1,25(OH)_2D_3$ and VDR in adipogenesis and lipid metabolism. Two studies investigated the metabolic phenotype of VDR-null mutant mice [28,29] in the Boston model [25]. In these mice the genetic alteration results in a frame shift followed by premature termination codon, which generates an apparent instability of the VDR transcript [25]. These animals exhibited decreased body weight, diminished body fat, reduced serum levels of leptin and lipids (triglycerides and cholesterol), while the calorimetric parameters such as energy expenditure, oxygen consumption and CO_2 production were increased. At the cellular level, beta-oxidation was higher in white adipose tissue (WAT), and the expression of carnitine palmitoyltransferase 2 (CPT2, the mitochondrial transporter of fatty acids) was increased in the knockout animals, compared to the wild type (WT) littermates [28]. An increase in the expression of all uncoupling proteins (UCP) in the brown adipose tissue (BAT) was also observed in the VDR

KO mice, suggesting an enhanced energy uncoupling in these cells and higher basal energy expenditure. Accordingly, treatment of brown adipocytes with $1,25(OH)_2D_3$ downregulated the expression of all three UCPs [28]. The inhibitory effect of VDR on UCP expression has been demonstrated in studies carried out in human adipose tissues [29] and in hereditary vitamin D-resistant rickets [30] establishing VDR as a modulator of energy balance in humans. The metabolic and molecular perturbations found in primary adipocytes and in the KO mice suggest key action of liganded VDR in the mitochondria and explain the lower adipose mass and lower plasma lipid profile in the mutant mice.

A similar lean phenotype was also described in mice lacking the 1α-hydroxylase enzyme that generates $1,25(OH)_2D_3$ [31], supporting the important role of $1,25(OH)_2D_3$ in lipid metabolism.

The lean phenotype found in VDR KO model has the same traits as the hyperthyroid metabolism described in humans and in mice models of hyperthyroidism [32-35]. This similarity could be explained by hypothesizing a competition between VDR and thyroid hormone receptors (THR) on cofactors or binding partners that would become limiting in the activity of the hormones. This could be the case for RXR alpha, the heterodimerization partner of many steroid receptors; the competition over RXR has been demonstrated not only for VDR and THR [36] but also for other members of the steroid/nuclear hormone receptor superfamily, such as peroxisome proliferator-activated receptors (PPAR) and liver X receptor (LXR) [37-40]. Another limiting candidate could be the coactivator DRIP 205/MED1 (vitamin D-interacting protein 205/mediator complex subunit 1), which is necessary for transcriptional activity of both VDR and THR [41,42]. Therefore the lean phenotype of VDR KO mice could be the result of a direct effect of VDR silencing and a potentiated action of thyroid hormone.

The observations made in VDR KO mice are confirmed by the opposite results obtained in the transgenic mouse model overexpressing the human VDR specifically in the adipose tissue [43], which gains adipose mass and reduces energy metabolism compared with the WT counterpart. In direct opposition to what described for VDR KO mice, in the transgenic model the investigators

found reduced leptin levels, increased circulating cholesterol and decreased calorimetric parameters. The overexpression of VDR in the adipocytes led to reduced UCP expression, decreased beta-oxidation and lipolysis, and glucose intolerance. The metabolic derangement was accompanied by suppression of key genes involved in these processes (hexokinase, carnitine palmitoyl transferases, lipases). Interestingly enough, when the effect of $1,25(OH)_2D_3$ was analysed in two adipocyte cell lines, a direct transcriptional control of these enzymes was not evident, rather suggesting that $1,25(OH)_2D_3$ influences fatty acid transport, catabolism and glycolysis by indirectly regulating these genes [43].

In conclusion, the analysis of VDR KO and transgenic mice demonstrated that the mitochondrial action of $1,25(OH)_2D_3$ is exerted on catabolism of fatty acids and uncoupling. $1,25(OH)_2D_3$ reduces lipid utilization from depots and decreases the energy and heat dissipation due to uncoupling process. As a consequence, VDR ablation leads to the lean phenotype. The precise mechanism through which VDR can control lipid catabolism remained elusive.

$1,25(OH)_2D_3$ controls lipid metabolism also in the skin. VDR KO mice have also defects in permeability barrier homeostasis due to a disrupted barrier formation, lipid secretion, and lipid composition [44]. The importance of $1,25(OH)_2D_3$ and VDR in the regulation of permeability barrier formation is supported also by the observations made in mice deficient in 1α-hydroxylase; these animals show an impaired barrier repair after acute disruption due to a decrease in lamellar body secretion [45].

4. The effects of $1,25(OH)_2D_3$ and VDR on mitochondrial activity: the consequences on lipid metabolism

Recent studies have described the influence of $1,25(OH)_2D_3$ on mitochondrial activity. A first work has demonstrated that in the human keratinocyte cell line HaCaT the VDR can downregulate the transcription of proteins of the respiratory chain and can decrease the mitochondrial membrane potential, in other words VDR restrains the activity of mitochondrial respiratory chain [21]. This

conclusion is supported by the data presented in another study [23] that measured cellular bioenergetics coupled with mitochondrial stress tests by a XF24 Extracellular Flux Analyzer. The study investigated mitochondrial respiration in $1,25(OH)_2D_3$-treated human brown adipocytes and demonstrated that $1,25(OH)_2D_3$ suppresses basal oxygen consumption rate (OCR), maximal OCR and OCR from proton leak. The metabolic modulation exerted by $1,25(OH)_2D_3$ /VDR sustains cell proliferation, and indeed several cancer cell lines silenced for VDR are inhibited in their growth [21]. One of the consequences of VDR-induced brake on respiratory chain is the rerouting of mitochondrial acetyl-CoA towards biosynthesis. In proliferating cells the diversion benefits mainly the cholesterol production necessary to proliferation [21], thus the metabolic modulation exerted by $1,25(OH)_2D_3$/VDR sustains cell growth, and indeed several cancer cell lines silenced for VDR are inhibited in their growth [21]. By contrast, in differentiated keratinocytes that upregulate the fatty acid biosynthetic pathway the metabolic switch promotes lipid deposition [22]. We can conclude that the effects of $1,25(OH)_2D_3$ on mitochondria support differently the metabolism of differentiated and proliferating human cells.

At present, with this novel insight into the modalities of $1,25(OH)_2D_3$ action, we can better understand the observations made in VDR KO and $1,25(OH)_2D_3$-deficient models by reinterpreting the metabolic data and formulating new hypothesis, summarized in figure 1. VDR downregulates respiratory chain activity, curtails the utilization of reducing units and slows down beta-oxidation. As consequence, the mitochondrial activity of VDR decreases lipid catabolism and promotes the channeling of acetyl-CoA into biosynthetic pathways (figure 1A). Indeed the consequences of VDR overexpression are the increased adipose mass and the reduced energy metabolism observed in the transgenic mouse model [43]. When VDR activity is lost, in knockout or diet-deficient animals, the mitochondrial respiratory activity is enhanced and UCPs expression is higher, leading to the observed increased calorimetric parameters [28,29]. The dual control of VDR on respiratory chain and UCPs expression is interesting and makes sense, in order to avoid unnecessary uncoupling and finely tune a proton gradient which cannot rely only on ATPase activity. Because of this concerted

control, the formation and dissipation of proton gradient is balanced. When VDR is ablated, the increased respiratory activity enhances the TCA cycle and beta-oxidation rises. The low levels of cytosolic malonil-CoA (derived from acetyl-CoA) relieve the inhibition of fatty acid mitochondrial import (figure 1B); as consequence, fat is mobilized from depots and catabolized in mitochondria. The cachectic phenotype observed in $1,25(OH)_2D_3$-deficient animals is another consequence of the increased oxidative metabolism, because muscle wasting is due to aminoacid utilization in TCA cycle.

5. $1,25(OH)_2D_3$ activity and adipogenesis

When the effects of $1,25(OH)_2D_3$ activity are investigated in vitro, both adipogenesis and adipocyte differentiation appear to be affected by the hormone, but the observations reach conflicting conclusions depending on the cellular model investigated.

The genome-wide studies based on next-generation sequencing have demonstrated the important role of histone modifications and epigenetic modulators in adipogenesis, and have added new details on how the cellular context (for example in response to thermogenic signals) can change the genomic programming of adipocytes (reviewed in [46]).

Also the genomic effects of $1,25(OH)_2D_3$ are strongly influenced by the cellular setting. The most recent genome-wide analysis of the $1,25(OH)_2D_3$-modulated transcriptome have hightlighted the complexity of events that determine the recruitment of VDR/RXR heterodimers and co-activators to binding sites often located in the enhancer regions of genes, and have described the interaction with general regulators of the chromatin remodeling process (reviewed in [47]). The VDR cistrome profile is lineage specific and it is modulated by the cellular status. For example the cellular differentiation dictates a remarkable quantitative and qualitative change of the transcriptional control exerted by $1,25(OH)_2D_3$. The studies on osteoblast differentiation [48-51] have described the reduction and modification of VDR-dependent transcriptome in differentiated cells: many genes become resistant to $1,25(OH)_2D_3$ stimulation and others change their transcriptional response; the

reasons of this plasticity related to cellular status can reside in the enrichment of transcription factors at nearby sites that can influence VDR activity and in the rearranged epigenetic landscape that can modify the access of VDR to binding motives close to target genes. In turn, bound VDR can influence the epigenetic profile of the cell and facilitate cellular transition [47,52].

Few studies have described the inhibitory effect of $1,25(OH)_2D_3$ on adipogenesis. The analysis of the mechanisms underpinning differentiation has been often carried out on the preadipocyte cellular model 3T3-L1 cell line, which can be terminally differentiated into mature adipocytes [53]. At time zero, the cells are treated with prodifferentiative agents (insulin, glucocorticoids, and phosphodiesterase inhibitor) that induce two rounds of cell division, and after two days the cells are switched to a differentiation medium containing only insulin. Several studies using the murine 3T3-L1 preadipocyte cell line have investigated the major transcriptional players in adipogenesis [54,55]. The early clonal expansion is driven by C/EBPβ (CCAAT/enhancer binding protein beta) [56] and glucocorticoid receptor, followed by a second cascade of transcription factors such as PPARγ, which is a primary driver of the adipocyte gene program. In the later stage the differentiated cells express the markers characteristic of adipocyte phenotype. $1,25(OH)_2D_3$ inhibits in a dose-dependent manner the early events of the adipogenic program (the first 48 hours), whereas it is ineffective if administered in the late phase of the differentiation protocol [2]. The effect is mediated by the stabilization of VDR protein, by the suppression of PPARγ upregulation and the antagonization of PPARγ activity [2, 57]. The activities of VDR and PPARγ are linked and influence each other. Indeed VDR overexpression suppresses PPARγ transactivation in brown preadipocytes [23] and this negative modulation has been reported for another member of the PPAR family [58]. On the other hand, the overexpression of PPARγ attenuates $1,25(OH)_2D_3$-mediated transactivation of VDR and competes with VDR for their binding partner RXRα [37]. Moreover $1,25(OH)_2D_3$ downregulates C/EBPβ gene expression in differentiating 3T3-L1 cells [1]. Other studies carried out in a line of brown adipocytes [23] and in the femoral bone marrow derived

cell line (BMS2) [59] confirmed that 1,25(OH)2D3 suppresses adipogenesis in the early phase of the differentiation process and the effect disappears if the cells are treated after day two of the differentiation protocol. Based on these observations we can conclude that $1,25(OH)_2D_3$ acts as anti-differentiating agent only in the early events of a differentiation protocol applied to proliferating cell lines, by interfering with the activity of key transcription factors.

On the other hand, the data collected from primary models used in adipogenesis studies show that in primary cells committed to adipocyte lineage the $1,25(OH)_2D_3$ exerts a differentiating adipogenic effect. Few established models to study adipogenesis start from primary bone cultures in which progenitor cells are committed to several mesenchymally derived cell types. In freshly isolated fetal rat calvaria (RC) cell populations the adipocyte differentiation is stimulated by $1,25(OH)_2D_3$ [60,61]. Moreover the treatment with the hormone increases the frequency of osteoblast/adipocyte bipotential progenitors and the number of colonies stained for lipids [62]. The primary bone marrow stromal cells are sensitive to $1,25(OH)_2D_3$ treatment that stimulates adipocytic cell differentiation [63]. $1,25(OH)_2D_3$ stimulates adipogenesis also in mesenchymal progenitor cells derived from normal human adipose tissue [64]. Microarray analysis of the adipogenic process showed that nuclear signaling of VDR, while promoting adipogenic differentiation, downregulates genes involved in differentiation into other lineages [64].

In conclusion, the effect of $1,25(OH)_2D_3$ on adipogenesis is opposite whether the cellular model analysed is a proliferating preadipocyte cell line or a committed primary culture of progenitor cells. The differences between the models can be ascribed to the lineage-specific chromatin remodeling taking place during differentiation protocols, which can determine the variability of VDR cistrome. A different recruitment of VDR and co-activators to DNA can have opposite effects on gene transcription. In addition to the genomic effects of its receptor, we must also consider the results of $1,25(OH)_2D_3$ activity on cellular metabolism. Based on the observation that the effects of $1,25(OH)_2D_3$ on mitochondria support differently the metabolism of differentiated and proliferating human keratinocytes [21,22], as discussed in section four, similar conclusions can be drawn on

adipogenesis, keeping in mind that the metabolic effects of $1,25(OH)_2D_3$ sustain the nuclear programming. We propose an integrated genomic and metabolic view that can conciliate the dual effect of $1,25(OH)_2D_3$ on adipogenesis; in our model, depicted in figure 2, the chromatin reorganization triggered by environmental clues influences VDR cistrome, so that VDR is effective on selected genes required by the cellular context, either proliferation or differentiation. At the same time, the effects of $1,25(OH)_2D_3$ on mitochondrial metabolism support the specific gene expression profile. When the adipogenesis of a preadipocyte cell line is considered, in the early phase of a differentiation protocol the stimulation with glucocorticoids, insulin and phosphodiesterase inhibitor maintains a proliferative asset; in this phase $1,25(OH)_2D_3$ sustains proliferation since acetyl-CoA can be diverted toward cholesterol biosynthesis. As consequence, in this model the outcome of $1,25(OH)_2D_3$ treatment in the early phase of differentiation is a decreased lipid deposition (figure 2A). In the late events of differentiation, when the cultural conditions change and adipogenesis is consolidated, the treatment with $1,25(OH)_2D_3$ can support the differentiated state by diverting acetyl-CoA into fatty acid biosynthesis. The VDR seems necessary for lipid accumulation in the late phase, since knock-down of the VDR both delays and prevents this process [1,64]. This happens despite the low levels of VDR found in differentiated 3T3-L1 cells [1,2], suggesting that even barely detectable amount of the receptor are sufficient and necessary to support the adipogenic function.

Instead, when the differentiation protocol is applied to primary cultures from bone, a model in which progenitor cells are committed to the adipocyte lineage, 1,25(OH)2D3 supports a nuclear differentiation program and facilitates lipid deposition because the mitochondrial acetyl-CoA can be used in the fatty acid biosynthetic pathway (figure 2B). In all cases, the metabolic response to $1,25(OH)_2D_3$ /VDR activity is the increased availability of acetyl-CoA for biosynthetic purposes, but the epigenetic reprogramming could be responsible for the biosynthetic fate of this precursor: for example the prevalence of SREBP-1 or SREBP-2 activity due to chromatin remodeling could potentiate lipogenesis or cholesterologenesis, respectively. In fact several data suggest that SREBP-

1 primarily regulates fatty acid metabolism and that SREBP-2 is the main regulator of cholesterol metabolism [65]. Few studies reported a differential accumulation of SREBP-1 or SREBP-2 under different dietary conditions consistently with each factor performing a preferred metabolic function in lipogenesis or cholesterologenesis in the liver [66-68]. A comparison of the genome-wide binding profiles for SREBP-1 and SREBP-2 in hepatic chromatin revealed that only 11.7 % of their binding sites overlap and demonstrated that under specific conditions they bind mostly to different sets of target sites in the liver genome [69]. Future genome-wide studies should integrate the VDR cistrome analysis with the examination of the binding profile of the key transcription factors in adipogenesis.

6. $1,25(OH)_2D_3$ deficiency in murine models and men: cachectic versus obese phenotype

The lean phenotype observed in VDR KO animals is found also in rats fed with a $1,25(OH)_2D_3$-deficient diet [70]. The two models show overlapping parameters, although with few differences due to calcium imbalance. In fact, whereas VDR KO animals are fed a diet enriched in calcium, indeed to avoid indirect metabolic effects, the dietary lack of $1,25(OH)_2D_3$ induces a perturbed calcium homeostasis that makes results difficult to interpret; the addition of calcium in this diet reverts partially the effects and adds new metabolic variables, such as the increase in insulin levels. The common features of animals deficient either in $1,25(OH)_2D_3$ or its receptor are the decreased body weight and fat, the reduced levels of leptin and increased expression of uncoupling proteins. Whereas in VDR KO is evident the enhanced utilization of fat from depots, in $1,25(OH)_2D_3$-deficient animals there are also the signs of reduced adipogenesis, because the transcription of PPARγ and fatty acid synthase is decreased [70]. $1,25(OH)_2D_3$ deficiency affects also muscular tissues, which decrease their mass similarly to fat depots. Muscle wasting in $1,25(OH)_2D_3$-deficient rats is due to increased muscle protein breakdown [71]. Therefore $1,25(OH)_2D_3$ deficiency leads to an unhealthy lean and cachectic phenotype characterized by muscle wasting and reduced adiposity.

If low levels of $1,25(OH)_2D_3$ or its receptor lead to the lean phenotype, it would be reasonable to expect the opposite result when a diet is fortified with $1,25(OH)_2D_3$. On the other hand, $1,25(OH)_2D_3$ increases calcium absorption and enhances intracellular calcium utilization, and this could stimulate the cellular energy expenditure, could activate the basal metabolism and facilitate a lean phenotype. The effects of $1,25(OH)_2D_3$ supplementation have been investigated in models of obesity induced by the high fat diet. These studies have reached conflicting conclusions, also because different animal models and different supplemented diets have been used. The long-term (one year) feeding of mice with a diet high in fat and low in vitamin D_3 (the precursor of $1,25(OH)_2D_3$), calcium, methyl donors and fiber leads to reduced energy expenditure and oxygen consumption, impaired glucose clearance and an elevated systemic inflammatory response. In this model, elevating dietary vitamin D_3 and calcium increases weight gain, fat deposition, and worsens the metabolic syndrome [72]. In contrast, both a mice and a rat model fed for a shorter time (ten or eight weeks) with a high fat diet benefit from $1,25(OH)_2D_3$ treatment, since $1,25(OH)_2D_3$ supplementation in mice protects against diet-induced obesity, improves glucose homeostasis and lipid oxidation [73], and reduces body weight gain, inhibits lipogenesis and promotes fatty acid oxidation in rat liver [74].

Clearly, in humans it is not possible to reproduce a VDR KO model. However, a primary deficiency or the resistance to $1,25(OH)_2D_3$ is found in some diseases: rickets, for example; unfortunately the lipid metabolism has not been investigated in these subjects.

Clinical studies correlate obesity with $1,25(OH)_2D_3$ deficiency [75-81]; this link is possibly due to a reduced synthesis of the active form of vitamin D in obese individuals, or to the sequestration of $1,25(OH)_2D_3$ in the increased fat tissue found in obesity. Indeed there are evidences that obesity is characterized by a decreased expression of the two biosynthetic enzymes of $1,25(OH)_2D_3$ metabolism, the 25-hydroxylase and the 1α-hydroxylase, in human adipose tissue [82]. Moreover the high fat diet in mice resulted in a decrease of 25(OH)-vitamin D plasma level [73]. Therefore it seems reasonable to hypothesize that obesity and metabolic syndrome are promoters of

$1,25(OH)_2D_3$ deficiency, although it is unknown the mechanism by which the perturbed lipid homeostasis would affect the biosynthetic enzymes. This view is endorsed by the recent bi-directional Mendelian randomization analysis of 21 adult cohorts, which has demonstrated that higher BMI (body mass index) leads to lower $1,25(OH)_2D_3$ status, whereas there is no evidence for a causal role of $1,25(OH)_2D_3$ deficiency in the development of obesity [83]. The fact that low levels of $1,25(OH)_2D_3$ are not causative of obesity is supported by the irrelevance of $1,25(OH)_2D_3$ supplementation in clinical trials of obese patients [84-87].

7. Concluding remarks and future perspectives

Recent studies have highlighted the relevance of $1,25(OH)_2D_3$ activity on mitochondrial metabolism, suggesting that many previously described metabolic effects of $1,25(OH)_2D_3$ on adipose tissue could be the result of mitochondrial modulation.

$1,25(OH)_2D_3$ exerts a concerted action on nuclear and mitochondrial compartment, and facilitates the adaptation of the mitochondrial metabolism to the phenotype generated by nuclear inputs. The consequences of $1,25(OH)_2D_3$ action on adipogenesis are dual, because the hormone can support both the proliferative phase and the differentiation process of the precursors of adipocytes.

In differentiated tissues $1,25(OH)_2D_3$ sustains a physiological mitochondrial lipid metabolism by two main mechanisms:

(1) the vitamin D-dependent calcium uptake in tissues enhances lipid catabolism. In fact it has been demonstrated that high dietary calcium increases fat oxidation rate and that low calcium intake can be associated with higher body fatness (reviewed in [88]). $1,25(OH)_2D_3$ stimulates intestinal calcium absorption and renal calcium reabsorption, in order to maintain the serum levels of calcium. Also calcium utilization in tissues is influenced by the hormone. Actually many of the metabolic effects of $1,25(OH)_2D_3$ are mediated by calcium, as demonstrated by the fact that most of the changes observed in $1,25(OH)_2D_3$ deficient rats were corrected by calcium supplementation alone [70]. In support of the calcium-mediated beneficial effects of 1,25(OH)2D3 , few studies have reported that

the supplementation with $1,25(OH)_2D_3$ in association with calcium decreases fat mass in obese patients [89,90] and affects glucose metabolism and lipid concentrations in overweight and obese vitamin D deficient women [91].

(2) $1,25(OH)_2D_3$ exerts a negative transcriptional control on respiratory chain elements and uncoupling proteins, in order to withhold respiratory activity and minimize the uncoupling process; thus by optimizing the respiratory performance $1,25(OH)_2D_3$ avoids the unnecessary catabolic fuelling that would lead to an unhealthy lean cachectic phenotype. This negative control of respiratory activity could be superimposed on the antagonism between VDR and THR. Their competition for binding partners and co-activators would make their alternation beneficial to the optimal respiratory rate.

In conclusion, $1,25(OH)_2D_3$ is necessary to accumulate lipids and to burn them properly. The effects of $1,25(OH)_2D_3$ supplementation could be different depending on dietary calcium content plus the individual metabolic phenotype and hormonal balance. Under this perspective, it will be very important for future therapeutic strategies to understand what are the causes of reduced biosynthesis of $1,25(OH)_2D_3$ in obesity and why $1,25(OH)_2D_3$ is scarcely effective in reducing fat depots in obesity.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legend

Figure 1. Schematic presentation of the consequences of mitochondrial activity of VDR on lipid metabolism. (A) VDR overexpression downregulates electron transport chain (ETC) and uncoupling protein (UCP) expression; the decreased utilization of reducing units NADH and $FADH_2$ slows down beta-oxidation. Mitochondrial activity of VDR decreases lipid catabolism and promotes the channeling of acetyl-CoA into biosynthetic pathways, whilst the mitochondrial import of fatty acids (FA) through carnitine acyltransferase (CAT) is inhibited by the biosynthetic precursor malonyl-CoA. (B) The loss of VDR activity reinforces ETC activity and UCP expression; TCA cycle and beta-oxidation are enhanced to fuel the boosted respiratory chain. When most of the mitochondrial acetyl-CoA is consumed by TCA cycle, very low levels are available in cytosol for acetyl-CoA carboxylase to synthesize malonil-CoA, the precursor of the biosynthetic pathway of lipids. The transport of fatty acid toward mitochondrial matrix increases because malonil-CoA no longer inhibits CAT. AS: ATP synthase.

Figure 2. The dual effect of $1,25(OH)_2D_3$ on adipogenesis. The mitochondrial effects of $1,25(OH)_2D_3$ support the nuclear program. (A) In proliferating preadipocyte cell line, in the early phase of a differentiation protocol the cultural stimulation maintains the cells in a proliferative condition and VDR cistrome adapts to the epigenetic profile imposed by the proliferative context. In addition, the mitochondrial effects of $1,25(OH)_2D_3$ support cell growth by shifting acetyl-CoA from the oxidative metabolism toward cholesterol biosynthesis. As consequence, $1,25(OH)_2D_3$ treatment in the early phase of differentiation facilitates proliferation and decreases lipid deposition. (B) In primary cultures of cells that are committed to the adipocyte lineage, VDR fits the chromatin remodeling induced by the differentiation program and modulates differentiation-specific gene sets. In this genomic background that promotes adipogenesis, $1,25(OH)_2D_3$ enhances lipid deposition because the mitochondrial acetyl-CoA can be diverted toward the synthesis of fatty acids. ETC: electron transport chain; AS: ATP synthase.

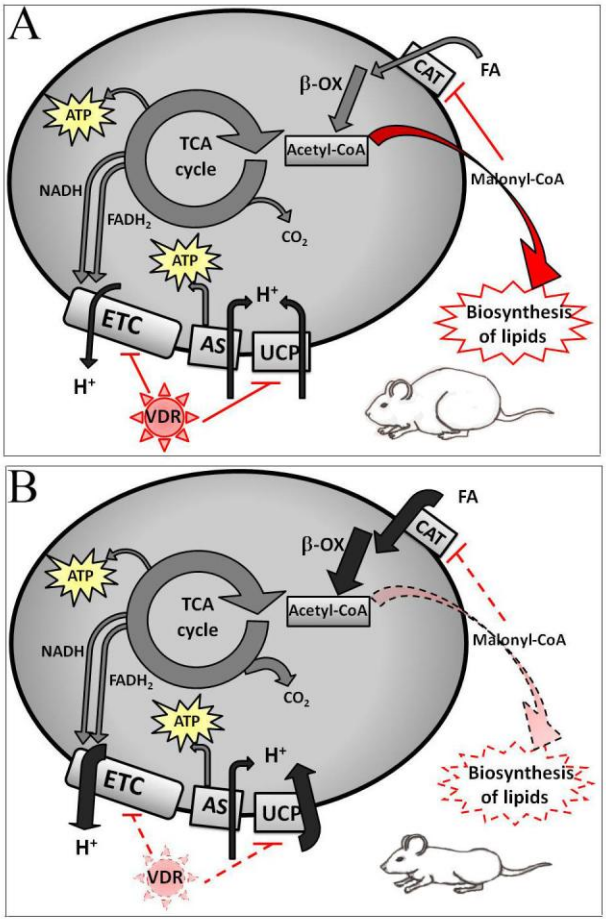

Figure 1

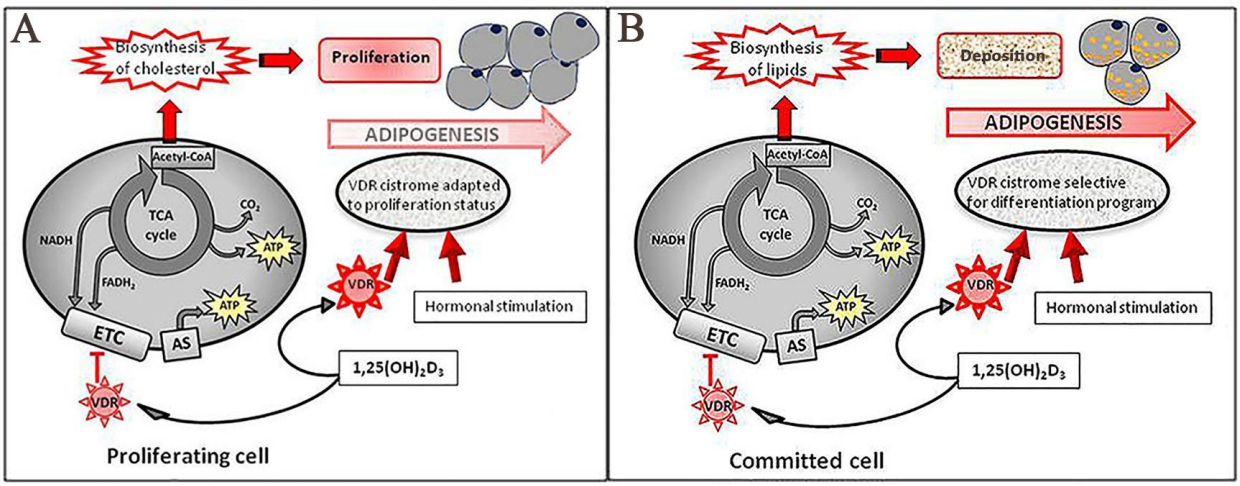

Figure 2