Mechanisms of vitamin D action in skeletal muscle

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Abstract

Vitamin D receptor expression and associated function have been reported in various muscle models, including C₂C₁₂, L6 cell lines and primary human skeletal muscle cells. It is believed that 1,25-hydroxyvitamin D₃ (1,25(OH)₂D₃), the active form of vitamin D, has a direct regulatory role in skeletal muscle function, where it participates in myogenesis, cell proliferation, differentiation, regulation of protein synthesis and mitochondrial metabolism through activation of various cellular signalling cascades, including the mitogen-activated protein kinase pathway(s). It has also been suggested that 1,25(OH)₂D₃ and its associated receptor have genomic targets, resulting in regulation of gene expression, as well as non-genomic functions that can alter cellular behaviour through binding and modification of targets not directly associated with transcriptional regulation. The molecular mechanisms of vitamin D signalling, however, have not been fully clarified. Vitamin D inadequacy or deficiency is associated with muscle fibre atrophy, increased risk of chronic musculoskeletal pain, sarcopenia and associated falls, and may also decrease RMR. The main purpose of the present review is to describe the molecular role of vitamin D in skeletal muscle tissue function and metabolism, specifically in relation to proliferation, differentiation and protein synthesis processes. In addition, the present review also includes discussion of possible genomic and non-genomic pathways of vitamin D action.

Key words: Calcitriol; Muscle; Mitochondria; Myogenesis; Protein synthesis

Introduction

The ‘sunshine hormone’, vitamin D, is a pro-steroid hormone that is reported to be the earliest hormone to arise on earth[1]. In the last 20 years, the number of scientific studies reporting the importance of vitamin D dietary intake and supplementation for cell and tissue function has increased dramatically. However, clinical studies have indicated that more than 1 billion individuals present with vitamin D insufficiency or deficiency[2–5]. Vitamin D receptor (VDR) expression in human muscle declines with age and a reduced capacity for UV-mediated vitamin D synthesis in the elderly’s skin may partly explain why the muscles of these individuals tend to be more susceptible to low vitamin D levels[6]. Hence, low vitamin D status has been considered a worldwide public health problem and is associated with the development of many diseases, such as osteoporosis, cancer, infertility, type 2 diabetes mellitus, coronary artery disease, and also has a significant impact on the immune system[7–10].

It has long been accepted that vitamin D plays a critical role in the regulation of Ca²⁺ and phosphate homeostasis and therefore is critical for impact on bone function. However, the discovery of a VDR in skeletal muscle cells provided further evidence of the important role of this hormone in skeletal muscle function and metabolism[11–14]. Low levels of vitamin D are associated with skeletal muscle fibre atrophy, muscle pain, weakness, and increased risk of sarcopenia and associated falls, in active and non-active individuals[15–18]. In athletes, low vitamin D levels are associated with poor bone health, and can impair muscle and immune functions, resulting in low muscle regenerative capacity after exercise sessions[19] and high risk of upper respiratory tract infections[20], respectively. The importance of vitamin D for skeletal muscle function and ultimately for whole body health is also supported by several randomised controlled trials (RCT) where vitamin D supplementation resulted in an increase in muscle strength in physically active and non-active individuals[21,22].

Several molecular mechanisms have been proposed to mediate the effects of vitamin D in muscle strength, function and metabolism, including changes in protein synthesis, myogenesis, mitochondrial activity, muscle regeneration and glucose metabolism[22–25]. However, the exact underlying mechanisms of vitamin D-related pathways, as well as their regulation and action in skeletal muscle, or how these pathways can be translated into clinical improvements, are unclear. Hence, the main purpose of the

Abbreviations: 1,25(OH)₂D₃, 1,25-hydroxyvitamin D₃; 25(OH)D, 25-hydroxyvitamin D; Akt, protein kinase B; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; MEF, myocyte enhancer factor; MRF, myogenic regulatory factor; mTOR, mammalian target of rapamycin; Myf5, myogenic factor 5; MyoD, myoblast determination protein; MYOG, myogenin; PKB, protein kinase B; RCT, randomised controlled trial; TGF-β, transforming growth factor β; VDR, vitamin D receptor.

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present review is to discuss the evidence for vitamin D action in skeletal muscle function and metabolism, at the molecular level, citing results from studies in vitro, in vivo and clinical research.

**Vitamin D physiology and synthesis**

Vitamin D is a liposoluble pro-hormone mostly found in two forms (vitamin D2 and vitamin D3) and is obtained by a self-regulated process, as described below. Sources of dietary vitamin D include oily fish, eggs and dairy products; however, the consumption and intestinal absorption of vitamin D represent only a minor part of the total vitamin D requirement for the whole body\(^{15}\). Exposure of the skin to UV radiation (wavelength 290–315 nm) is associated with 80–90% of total vitamin D synthesis and allows 7-dehydrocholesterol to be converted to cholecalciferol (pre-vitamin D3)(15,26,27). Both endogenous synthesis and exogenous dietary intake raise serum concentrations of vitamin D, which undergoes two hydroxylation steps to the final product\(^{28}\). Firstly, pre-vitamin D3 is thermal isomerised to vitamin D3, binds to vitamin D binding protein and is subsequently transported into the liver where it is hydroxylated by the enzyme vitamin D 25-hydroxylase to 25-hydroxyvitamin D (25(OH)D). This molecule is the predominant circulating form of vitamin D that is traditionally measured to identify vitamin D status\(^{29}\). 25(OH)D is used as a reliable marker of vitamin D status because it has a dominant circulating form of vitamin D that is traditionally measured in serum, with a half-life of 19 days\(^{30}\). The biologically active form 1,25(OH)2D3 is responsible for acting with its heterodimeric partner, the retinoid X receptor, in the regions of DNA and activate or inhibit gene transcription\(^{31}\). The 1,25(OH)2D3 can be considered a different pathway for regulation of myogenesis such as Myf5, myostatin, atrophy marker E3-ubiquitin ligases and muscle ring-finger protein-1 (MRF)\(^{36}\). Interestingly, in many studies the expression of MRF signalling molecules induce the activation of skeletal muscle cell receptors and regulate transcription of specific target genes in order to develop the adult skeletal muscle tissue, such as SIX1–SIX6 and myocyte enhancer factor (MEF)2 proteins (MEF2A, MEF2C and MEF2D)\(^{42}\).

**Role of vitamin D in skeletal muscle**

**Proliferation and differentiation**

The development of muscular tissue (myogenesis) has several phases starting from stem cells located in somites, followed by the development of the first progenitor cells named myoblasts, and finally their differentiation into mature myotubes\(^{39}\). Regulation of myogenesis depends mainly on two transcription factor systems: paired-box transcription factors, Pax3 and Pax7, and a family of basic Helix–Loop–Helix transcription factors known as myogenic regulatory factors (MRFs)\(^{40}\). Myoblasts then proliferate extensively until they reach a myofibrillar protein synthesis peak and then differentiate into mature myotubes, due to activation of MRF. To maintain tissue homeostasis, there is a subpopulation of cells that resides in the quiescent state, also known as satellite cells. These cells also have the potential to differentiate into new muscle fibres (i.e. myogenesis) and maintain protein turnover\(^{41,42}\). Briefly, the fate and differentiation of muscle cells are controlled by four MRF: myogenic factor 5 (Myf5), muscle-specific regulatory factor 4 (MRF4), myoblast determination protein (MyoD) and myogenin (MYOG). Myf5 and MyoD are specifically degraded at mitosis and G1/S, respectively, and this is mediated by phosphorylation via cyclin-dependent kinases. Myf5 and MyoD determine skeletal muscle cell identity, and are consequently considered to be the gatekeepers for entry into the terminal specification of myogenic lineage\(^{43}\), whereas MYOG is essential for the differentiation of myoblasts into myotubes\(^{44}\). MYOG acts genetically downstream of MyoD and Myf5 to switch on muscle differentiation genes. Although MRF4 is classified as a differentiation gene, it is also believed to act as a determinant gene when it is expressed by undifferentiated cells\(^{45}\). MRF signalling molecules induce the activation of skeletal muscle cell receptors and regulate transcription of specific target genes in order to develop the adult skeletal muscle tissue, such as SIX1–SIX6 and myocyte enhancer factor (MEF)2 proteins (MEF2A, MEF2C and MEF2D)\(^{42}\).

Several animal studies have demonstrated that a vitamin D-deficient diet decreases markers of proliferation, such as bone morphogenetic protein family, fibroblast growth factor 2 and/or proliferating cell nuclear antigen, while increasing markers of myogenic differentiation such as Myf5, myostatin, atrophy marker E3-ubiquitin ligases and muscle ring-finger protein-1 (Table 1)\(^{46}\). Interestingly, in many studies the expression of VDR in skeletal muscle increases in animals receiving a vitamin D-deficient diet and/or vitamin D-rich diet, which suggests that the effects of vitamin D are dependent on its receptor\(^{47,48}\). VDR was also detected in the nucleus of muscle fibres from middle-aged and older female patients with osteoarthritis/osteoporosis and the expression seems to decrease with age\(^{49,50}\). In accordance with these studies, Ceglia et al.\(^{51}\) also confirmed the VDR expression in skeletal muscles from healthy
Table 1. Overview of the biomolecular role of vitamin D (VitD) in skeletal muscle (six animal studies)

<table>
<thead>
<tr>
<th>Study</th>
<th>Species, n</th>
<th>VitD form, dose, time and diet</th>
<th>Findings and effects</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srikuea et al. (2012)</td>
<td>CS7BL/6 mice aged 12 weeks, n = 4</td>
<td>No VitD treatment; regeneration (injection of 1.2 % BaCl₂ to tibialis anterior v. control group)</td>
<td>↑ Expression of VDR and CYP27B1 gene at day 7 of regeneration v. control (P &lt; 0.001)</td>
<td>Expression of VDR in skeletal muscle during repair and regeneration</td>
</tr>
<tr>
<td>Hutton et al. (2014)</td>
<td>Male, Ross 708 roaster chicks, n = 48–52 birds per group</td>
<td>5000 IU/kg diet with VitD₃ or VitD₂ + 25(OH)D₃, 49 d</td>
<td>↑ Myogenic factor 5, ↑ density of satellite cells (P &lt; 0.05), ↑ total nuclear density (P &lt; 0.05) and ↑ muscle fibre cross-sectional area (P &lt; 0.09) in pectoralis major muscle</td>
<td>Hypertrophic response in the pectoralis major muscle</td>
</tr>
<tr>
<td>Domingues-Faria et al.</td>
<td>15-month-old Wistar rats, n = 10 per group</td>
<td>AIN-93M maintenance diet (1000 IU VitD₃/kg) or without VitD for 9 months</td>
<td>↓ 74 % plasma 25(OH)D with VitD-deficient diet (P &lt; 0.01)</td>
<td>↓ Notch pathway activity and proliferation potential</td>
</tr>
<tr>
<td>Girgis et al. (2015)</td>
<td>CS7BL/6 mice, n = 6–12 per group</td>
<td>Deletion of VDR-KO v. wild-type littermates; Diet: 2.2 IU/g or VitD-deficient diet for 3 months</td>
<td>Weakener grip strength: VDR-KO and VitD-deficient mice (P &lt; 0.005)</td>
<td>Weakness advanced with age and duration of VitD deficiency</td>
</tr>
<tr>
<td>Ray et al. (2016)</td>
<td>Female A/J mice, n = 5 per group</td>
<td>AIN-93-based diets with 1000 or 10 000 IU VitD₃/kg diet for 6 weeks</td>
<td>Maximal diaphragm force, twitch force, and fibre cross-sectional area ↓ 26, 28 and 10 % (respectively) with VitD-deficient diet (P &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>Oku et al. (2016)</td>
<td>Sprague–Dawley strain male rats, n = 6</td>
<td>AIN-93 diet (1000 IU VitD₃/kg) Control, VitD restriction, high-fat diet and high-fat diet with VitD restriction for 14 d</td>
<td>VitD restriction: ↓ volume of the femur (P &lt; 0.001), ↓ bone mineral density (P &lt; 0.05), ↓ MyoD (P &lt; 0.05)</td>
<td>Muscle mass trending towards a ↓ in VitD restriction group (P &lt; 0.05)</td>
</tr>
</tbody>
</table>

1, Increase; ↓, decrease; 25(OH)D, 25-hydroxyvitamin D; AIN, American Institution of Nutrition; BMP4, bone morphogenetic protein family; FGF2, fibroblast growth factor 2; IU, international units; MyoD, family of myogenic regulatory factors; MuRF1, muscle ring-finger protein-1; VDR-KO, vitamin D receptor knockout mouse; PCNA, proliferating cell nuclear antigen; VDR, vitamin D receptor.

postmenopausal women. Conversely, a recent study has reported that VDR protein is readily detected in human myoblasts and myotubes, while non-detectable in adult human skeletal muscle tissue. It is important to highlight that the detection of VDR expressed in skeletal muscle cells and tissue varies according to the technique used for protein extraction and the type of primary antibody; consequently many authors have found difficulty in detecting this receptor.

The majority of animal studies have focused on the physiological effects of vitamin D on muscle mass and strength and most have not investigated the precise pathways regulating the reported outcomes. At the cellular level, some studies have investigated the effects of vitamin D in murine cell lines, such as C₂C₁₂ (Table 2), and demonstrated that treatment with vitamin D inhibits cell proliferation and stimulates cell differentiation. More recently, Braga et al. have shown that treatment with 1,25(OH)₂D₃ in primary cultures of satellite cells enhances myogenic differentiation through an increase in the expression of myogenic markers, such as MyoD and MYOG, myotube formation, and the modulation of pro- and anti-myogenic factors. These results are in agreement with previous studies that identified an increase in myogenic factors such as MYOG, Myf5 and MYC2 (transcription factor) in response to vitamin D treatment. In addition, Ryan et al. were the first group to prove a dose–response effect of the active form of vitamin D to modulate the capacity of C₂C₁₂ cells to transdifferentiate into adipocytes. Low concentrations of vitamin D (simulating a deficient status) induced adipogenesis and up-regulation of key adipogenic marker genes (PPARγ2 and fatty acid binding protein 4 (FABP4)), whereas higher concentrations attenuated the differentiation into adipocytes. Consequently, an increase in triacylglycerol synthesis and levels within skeletal muscle is associated with a decrease in functional strength and impairment of glucose tolerance, leading to a higher risk of developing metabolic diseases, insulin resistance, obesity and type 2 diabetes. However, in primary human skeletal muscle cells, the effects of vitamin D on proliferation and differentiation are conflicting. Owens et al. have investigated the effects of vitamin D during differentiation in human primary muscle cells collected by biopsies from active adults and found an increase in myotube fusion and differentiation. On the other hand, Olsson et al. reported opposite effects regarding differentiation, such as reduction of expression of cell cycle regulators and myogenic regulatory factors (MyoD, MYOG, MEF2C and sarcomeric proteins), with associated activation in forkhead box O3 and Notch signalling pathways.
An important factor to consider regarding skeletal muscle cell lines is that the conversion of 25(OH)D to its active form (1,25(OH)2D3) has not been confirmed to occur locally in primary skeletal muscle cells, whereas it does occur in the skeletal muscle murine cell line C2C12 and in vivo in mice(23,59). In addition, discrepancies exist among studies regarding the cell line used, and vitamin D form, dose and duration of the treatment, consequently leading to different outcomes. Overall, vitamin D influences myogenesis through regulation of myogenic factors and proteins involved in this process. The main findings suggest that vitamin D promotes differentiation while reducing proliferation in murine cells; however, is still difficult to define how vitamin D affects proliferation and differentiation in primary human muscle cells. Further studies are needed to elucidate the cellular development stages and/or subpopulations of cells that undergo regulation by vitamin D and thus affect the process of muscular development.

**Protein synthesis and myotube size**

Cellular protein content is controlled by anabolic and catabolic mechanisms that regulate synthesis and degradation of muscle proteins, resulting in changes in muscle mass(43). The main mechanisms that regulate protein synthesis in skeletal muscle

Table 2. Overview of the biomolecular role of vitamin D (VitD) in skeletal muscle cells (eleven in vitro studies)

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell line/cell type</th>
<th>VitD form, dose and time</th>
<th>Significant findings and effects</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garcia et al. (2011)(66)</td>
<td>C2C12 Myoblasts</td>
<td>100 nM-1,25(OH)2D3 1–10 d</td>
<td>↓ Proliferation, ↓ IGF-1, ↑ myogenic differentiation, ↑ IGF-2, ↓ follistatin expression and ↓ myostatin</td>
<td>2-fold ↓ in the mean diameter of the fibres (P&lt;0.001); 2.5-fold ↓ in size (width) (P&lt;0.001)</td>
</tr>
<tr>
<td>Srinuva et al. (2012)(77)</td>
<td>C2C12 Myoblasts, myotubes</td>
<td>20 nM-1,25(OH)2D3 or 2 μM-25(OH)2D3 5 d</td>
<td>Inhibits cell proliferation (P&lt;0.001)</td>
<td>↓ Myoblast number after treatment</td>
</tr>
<tr>
<td>Garcia et al. (2013)(68)</td>
<td>C2C12 Myoblasts</td>
<td>100 nM-1,25(OH)2D3 1, 3, 4, 7 or 10 d</td>
<td>↓ Proliferation (FGF2 and TIMP-3) (P&lt;0.05)</td>
<td>↓ Myostatin expression (negative regulator of skeletal muscle mass)</td>
</tr>
<tr>
<td>Salles et al. (2013)(72)</td>
<td>C2C12 Myotubes</td>
<td>0, 1 or 10 nM-1,25(OH)2D3 72 h</td>
<td>125(OH)2D3 (10 nM) + leucine and insulin, ↑ protein fractional synthesis rate (14–16 %) (P&lt;0.01)</td>
<td>Akt/mTOR-dependent pathway was enhanced by 1,25(OH)2D3</td>
</tr>
<tr>
<td>Girgis et al. (2014)(69)</td>
<td>C2C12 Myoblasts, myotubes</td>
<td>100 nM-1,25(OH)2D3, 100 nM-25(OH)D 48 h</td>
<td>Proliferation and differentiation (↑ myostatin, myc, myogenin, myogenic factor 5 and cyclin-D; ↑ retinoblastoma protein and ATM) (P&lt;0.05)</td>
<td>1.8-fold ↑ in cross-sectional size of individual myotubes</td>
</tr>
<tr>
<td>Irazoqui et al. (2014)(66)</td>
<td>C2C12 Myoblasts</td>
<td>C2C12 wild-type or VDR (knockdown) 1 mM-1,25(OH)2D3 6, 12 and 24 h</td>
<td>Induces differentiation (↑ myogenin) (P&lt;0.05)</td>
<td>p38-dependent co-localisation of VDR and cyclin D3</td>
</tr>
<tr>
<td>Owens et al. (2015)(25)</td>
<td>Human myoblasts and myotubes (biopsy)</td>
<td>10 or 100 nM-1,25(OH)2D3 7–10 d</td>
<td>With 10 nM-1,25(OH)2D3, ↑ muscle cell migration, dynamics and ↑ myotube fusion/differentiation</td>
<td>Humans: improves recovery of peak torque at 48 h and 7 d post-exercise</td>
</tr>
<tr>
<td>Ryan et al. (2016)(24)</td>
<td>Human myoblasts</td>
<td>10 nM-1,25(OH)2D3 48 h</td>
<td>↑ Mitochondrial oxygen consumption rate (P&lt;0.003)</td>
<td>↑ Mitochondrial volume and branching</td>
</tr>
<tr>
<td>Van der Meijden et al. (2016)(63)</td>
<td>C2C12 Myoblasts,myotubes</td>
<td>0, 400 or 1000 nM-25(OH)D or 1,25(OH)2D3 24 h</td>
<td>↑ VDR mRNA expression and ↓ proliferation (P&lt;0.01)</td>
<td>C2C12 is able to metabolise 25(OH)D3 and 1,25(OH)2D3</td>
</tr>
<tr>
<td>Olsson et al. (2016)(23)</td>
<td>Human myoblasts and myotubes (biopsy)</td>
<td>1 or 100 nM-1,25(OH)2D3 48 h</td>
<td>Lack of ability to convert 25(OH)D into 1,25(OH)2D3 Inhibits myoblast proliferation and differentiation (P&lt;0.014 and P&lt;0.012)</td>
<td>Changes in forehead box Q3 and Notch signalling pathways</td>
</tr>
<tr>
<td>Braga et al. (2017)(61)</td>
<td>Mouse skeletal muscle Satellite cells</td>
<td>100 nM-1,25(OH)2D3 1–12 d</td>
<td>↑ Expression: MyoD, MYC2, myogenin, skeletal muscle fast troponin I and T, MYH1, IGF-1 and -2, FGF1 and 2, BMP4, MMP9 and follistatin (P&lt;0.05)</td>
<td>↑ Myotube formation and expression of myostatin (P&lt;0.001)</td>
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</table>
are via the insulin signalling cascade, insulin-like growth factors (IGF, such as IGF-1 and IGF-2) and amino acids\(^{66}\). These molecules, upon receptor binding, induce phosphorylation and activation of sequential targets, including the insulin receptor substrate, phosphatidylinositol-3 kinase (PI3K), phosphoinositide-dependent kinase-1, protein kinase B serine/threonine kinase family (Akt/PKB), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (p70S6k)\(^{67}\).

mTOR is the major regulator of cell growth and proliferation by controlling the initiation phase of protein translation and synthesis (Fig. 1)\(^{68}\). Furthermore, it has been suggested that regulatory proteins, for example transforming growth factor \(\beta\) (TGF-\(\beta\)) family and myostatin, also play an essential role in protein synthesis and myotube size by regulating Akt/PKB, mitogen-activated protein kinase (MAPK) and mTOR pathways\(^{69,70}\). The proposed model of signalling is via activation of the TGF-\(\beta\) family and myostatin, also play an essential role in protein synthesis and myotube size by regulating Akt/PKB, mitogen-activated protein kinase (MAPK) and mTOR pathways\(^{69,70}\). The proposed model of signalling is via activation of the TGF-\(\beta\) family and myostatin, also play an essential role in protein synthesis and myotube size by regulating Akt/PKB, mitogen-activated protein kinase (MAPK) and mTOR pathways\(^{69,70}\). The proposed model of signalling is via activation of the TGF-\(\beta\) family and myostatin, also play an essential role in protein synthesis and myotube size by regulating Akt/PKB, mitogen-activated protein kinase (MAPK) and mTOR pathways\(^{69,70}\).

The first in vitro study to suggest an association between vitamin D and improvements in net muscle protein synthesis was published in 1975\(^{71}\). This first observation indicated that 25(OH)D supplementation increased \(^{3}H\)leucine incorporation into proteins in rat skeletal muscle and specified that the vitamin D effect was, at least in part, independent of the action of the intestinal transport of Ca and phosphate\(^{71}\). However, Ca\(^{2+}\) and phosphate (PO\(_4\)\(^{2-}\)) could be directly involved in the mechanism of action of vitamin D in muscle cells. Subsequent studies with VDR knockout (VDR-KO) mice, and with also mice subjected to a vitamin D-deficient diet (Table 1), have reported significant muscular atrophy, weakened strength, decreased muscle fibre size, lower bone mineral density and dysregulation of myogenic regulatory factors when compared with control groups, i.e. normal mice and mice receiving a vitamin D-sufficient diet\(^{46,53–55}\). Moreover, Oku et al.\(^{53}\) have demonstrated the negative impact of vitamin D deficiency on the levels of mRNA expression of MyoD in skeletal muscle tissue, which corroborates the importance of vitamin D for the process of myogenesis, muscle maintenance and hypertrophy. Salles et al.\(^{72}\) later confirmed that an in vitro treatment with 10 nmol\(\cdot\)L\(^{-1}\)25(OH)\(_2\)D\(_3\) potentiated the effects of leucine and insulin; and increased the protein synthesis rate by 14–16 % through Akt/PKB and mTOR pathways. In the later study vitamin D has also been shown to enhance the phosphorylation of Akt/PKB and glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)), and consequently improve insulin signalling by up-regulating the insulin receptor. It is proposed that Akt inhibits GSK3 and, as a consequence, activates the eukaryotic translation initiation factor 2B (eIF2B), resulting in the formation of the 43S preinitiation...
complex. Furthermore, mTOR phosphorylates eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and p70S6K allowing assembly of the 43S pre-initiation complex(73,74). Further research is important to identify if vitamin D has a biological effect on amino acid transporters, as a potential alternative pathway to improve protein synthesis and myotube size. Studies also have reported the action of vitamin D on muscle proteolytic activity and regulation via proteases, specifically the ubiquitin–proteasome pathway(75,76). In a rat muscle model, vitamin D deficiency led to an increase in activities of the glutathione-dependent enzymes and decrease in superoxide dismutase and catalase enzymes, resulting in oxidative stress and proteolysis. Rehabilitation with vitamin D could reverse the alterations in oxidative stress parameters, increase total protein degradation and muscle atrophy gene markers post-vitamin D treatment in C2C12 muscle cells(77). The same authors have also demonstrated an increase in protein degradation and decreased protein synthesis after a vitamin D-deficient diet in male rats(78).

Some of the strongest evidence linking vitamin D to protein synthesis is the stimulation of fibre hypertrophy(42). In one study, fibre hypertrophy was observed after 10 d of treatment with 1,25(OH)2D3, with a significant 2-fold increase in the mean diameter of C2C12 fibres, and an increase of 2-5-fold in length(56). These results were attributed to an increase in protein synthesis; however, the authors did not measure this parameter directly. Similar results were obtained by van der Meijden et al.(59) where the treatment with 25(OH)D for 3 d resulted in a 19% increase in C2C12 fibre diameter. Despite previous evidence of the impact of vitamin D on metabolic signalling pathways and phenotype in skeletal muscle cells, there is currently no evidence regarding the effect of vitamin D on protein synthesis in primary human skeletal muscle cells. Finally, in clinical studies, vitamin D supplementation in healthy human subjects with low serum levels activates the VDR in skeletal muscle tissue, which appears to stimulate protein synthesis and improve muscle strength following an increase in the size and number of type II muscle fibres(5,78).

Mitochondrial metabolism

Adaptive mitochondrial function is essential for cellular homeostasis, especially in high energy-demanding skeletal muscle cells, as mitochondria are the main organs responsible for generating energy in the form of ATP(79). Typically, the effects of vitamin D are interrelated by its interaction with a nuclear VDR and is part of the nuclear receptor superfAMILY of ligand-activated transcription factors. VDR can also be translocated into the mitochondria of certain cell types, including the skeletal muscle cells, and potentially act directly on cellular bioenergetics(80). This evidence led researchers to consider that mitochondrial activity could potentially be modified by a diet containing sufficient levels of vitamin D and/or supplementation, which is thought to increase cellular metabolism. Interestingly, a few studies have indicated that vitamin D supplementation modulates mitochondrial activity and enhances ATP production at rest and during exercise(80,81). More recently, a positive association between vitamin D and RMR was observed in obese adults(20). The latter study demonstrated that for every 10 nmol/l increase in serum 25(OH)D, the RMR increased by 56-5 kJ/d(16). However, another study with a very short (1 week) period of vitamin D supplementation reported no influence on energy or substrate utilisation(82). Additional randomised clinical trials examining the influence of vitamin D on energy expenditure are therefore urgently needed.

The mechanism of vitamin D action in mitochondrial metabolism currently proposed is an increase in the expression of electron transport chain proteins and the tricarboxylic acid cycle enzymes via both genomic and non-genomic pathways(83). This theory was tested by Muñoz Garcia et al.(84) who investigated pathways related to the tricarboxylic acid cycle, oxidative phosphorylation and ATP synthesis in existing gene expression databases from multiple related monocyte models(84). In this study, genes associated with the electron transport chain activity and in the conversion of acetyl-CoA to CO2 were up-regulated by vitamin D in three immune cell types, the THP-1 monocyte cell line, monocyte derived dendritic cells and monocytes(84). The same pattern was observed in human peripheral blood mononuclear cells linking serum vitamin D status with specific markers of bioenergetic pathways(85).

With respect to skeletal muscle tissue, the precise function of vitamin D in relation to mitochondrial metabolism remains highly elusive. Ryan et al.(20) has investigated the effects of vitamin D on cellular bioenergetics in primary human muscle cells (undifferentiated myoblasts). The authors have demonstrated that mitochondrial oxygen consumption rate increased when cells were treated with vitamin D for 48 h and this response was dependent of the VDR(24). A possible mechanism for this is, an elevation in mitochondrial volume fraction and branching, which may result in mitochondrial fusion and biogenesis. Vitamin D treatment increased expression levels of MYC, MAPK13 and endothelial PAS domain-containing protein 1 mRNA (which encodes for a protein that regulates mitochondrial biogenesis)(24). In addition, mediators of mitochondrial fusion were altered. Specifically, OPA1 expression increased following vitamin D treatment, while mediators of mitochondrial fission (Fis1 and Drp1) were decreased(24). Furthermore, current evidence has illustrated that treatment with 0.1 nmol L-1 25(OH)D3 in human primary muscle improved mitochondrial morphology (volume and structure) and altered mRNA expression of pyruvate dehydrogenase kinase 4 and carnitine palmitoyltransferase 1 (CPT1), important genes that control muscle glucose and lipid metabolism(80). Vitamin D deficiency is known to impair muscle function and metabolism, where, in this case, skeletal muscle fibres are most likely to VDR ablation and to uptake cytoplasmic Ca2+ released from the sarcoplasmic reticulum during twitch responses(97). Experiments with chick muscles deficient in vitamin D observed changes in oxidative phosphorylation and a failure of muscle mitochondria to maintain Ca2+, leading to impairment of cellular metabolic homeostasis, increased reactive oxygen species and cytotoxicity due to mitochondrial dysfunction(89). Despite previous reports, there are still no studies that have reported whether the effects of vitamin D up-regulate or down-regulate genes and proteins associated with the tricarboxylic acid cycle and electron transport chain in primary skeletal muscle cells.
**Effects of vitamin D in skeletal muscle**

**Actions and targets of vitamin D**

The outcomes of vitamin D action appear to be dependent on interaction with a nuclear VDR and with the retinoid X receptor. This complex is able to up-regulate and/or down-regulate target genes by binding to regulatory sequences named vitamin D3 response elements (Fig. 1)\(^{(89)}\). It is estimated that approximately 3% of the human genome is regulated, directly and/or indirectly, by the vitamin D–endocrine system\(^{(90)}\). Genomic mechanisms may explain how supplementation of vitamin D influences, for instance, muscle hypertrophy in adults. Gene expression was evaluated using muscle biopsies in a retrospective cohort of healthy volunteers\(^{(111)}\). In this study, a positive correlation between serum active vitamin D and genes encoding for transcriptional activators or co-repressors of TGF-β and myostatin were observed, resulting in anti-proliferative effects\(^{(11)}\). Associations between vitamin D levels and up-regulation of gene expression were also found with molecules involved in protein synthesis pathways, such as IGF-1 receptor and eukaryotic translation initiation factor 4e-binding protein 1 (EIF4BP1) and eukaryotic translation initiation factor 2B subunit α (EIF2B1), which are members of a family of translation repressor proteins\(^{(11)}\). Additionally, it has been established that 1,25(OH)\(_2\)D\(_3\) regulates proteins that effect cell cycle and regeneration in a rat model of muscle crush injury\(^{(91)}\). In the present study, vitamin D treatment increased cell proliferation of non-satellite cells, including fibroblasts, endothelial cells, Pax7-negative local stem cells and prolyl-1-4-hydroxylase-β expression (P4HB), which have a direct impact on collagen synthesis. Treatment with vitamin D also increased the production of extracellular matrix components that can similarly affect muscle contractility and force\(^{(91)}\).

Another possible genomic mechanism of vitamin D involves the stimulation of IGF-1\(^{(92)}\). A study assessing rickets patients detected that circulating concentrations of IGF-1 increased significantly after vitamin D treatment and this may regulate the pituitary gland and growth hormone production\(^{(93)}\). More recently, a post hoc analysis of the Styrian Vitamin D Hypertension Trial did not find any significant effects of vitamin D supplementation on IGF-1 concentrations in hypertensive patients with low 25(OH)D levels at baseline; however, a significant effect was detected in a cross-sectional correlation between the active form of 1,25(OH)\(_2\)D\(_3\) and IGF-1\(^{(94)}\). Therefore, further studies are necessary to clarify the relationship between vitamin D and IGF-1/growth hormone. It is also known that vitamin D influences pathways of apoptosis in a variety of cells, including osteoblasts, osteocytes and tumour cells, resulting in anti-apoptotic effects\(^{(91)}\). These findings suggest that vitamin D acts via VDR/inositol triphosphate (IP\(_3\)) and Akt, consequently reducing caspase activity and increasing cell survival.

The typical steroid hormone (genomic pathway) is characterised by direct regulation of gene expression, which mediates the responses to the hormone some hours after hormone-receptor binding. In contrast, the non-genomic pathway is characterised by activation of a rapid second messenger which results in acute (mostly seconds) cellular responses and it is not dependent upon the immediate regulation of gene transcription\(^{(51)}\). 1,25(OH)\(_2\)D\(_3\) stimulates fast transcriptional-independent effects (seconds to minutes range) that are not feasibly explained by alterations in gene expression\(^{(17)}\). Although there is no consensus about the non-genomic pathways of vitamin D, studies have suggested that the actions of vitamin D start at the plasma membrane\(^{(95)}\). Initially, vitamin D binds to a surface or caveolar VDR, which activates c-Src and phosphoinositide-3 kinase and leads to the fast recruitment of Ca\(^{2+}\) from the sarcoplasmic reticulum into the cytosol. This results in the activation of phospholipase C\(_γ\) and release of IP\(_3\) and diacylglycerol from the membrane. Subsequently, IP\(_3\) facilitates the release of Ca\(^{2+}\) from the sarcoplasmic reticulum into the cytosol\(^{(96,97)}\).

Another non-genomic mechanism of vitamin D action is promotion of the translocation of PKC-α from the cytosol to the cell membrane, which has been previously studied using chick and rat myoblasts with *in vitro* 1,25(OH)\(_2\)D treatment (Fig. 1)\(^{(98,99)}\). PKC also activates the L-type voltage-dependent Ca\(^{2+}\) channel and store-operated Ca\(^{2+}\) entry (SOCE) channel and may have a role in the activation of extracellular signal-regulated kinases (ERK1/2). Briefly, studies have found a dose-dependent increase in intracellular muscle Ca\(^{2+}\) uptake after treatment with the active form of vitamin D, which may have an impact on muscle contraction\(^{(98,100,101)}\). In addition, the activation of c-Src results in Raf-1 stimulation\(^{(102,103)}\), which leads to the activation of the MAPK pathway. Subsequently, ERK1/2 activation and phosphorylation of ETS domain-containing protein (Elk-1) and cAMP response element-binding protein increases the expression of c-myc and c-fos, which are key regulators of proliferation and differentiation\(^{(104)}\). Finally, vitamin D also activates p38 MAPK and consequently phosphorylates heat-shock protein 27 which has a significant role in remodelling muscle cells through the actin microfilament system (Fig. 1)\(^{(105,106)}\). Even though this seems like a non-genomic effect of vitamin D through direct and acute stimulation of the AKT pathway, it is also known that both genomic and non-genomic effects are interdependent. For instance, extra nuclear-initiated actions may regulate gene expression indirectly through their effects in pathways that themselves control transcription\(^{(107)}\). New studies focusing on genomic and especially non-genomic mechanisms of vitamin D are required to better distinguish the precise contribution of each mechanism of action and optimal dose-effect for skeletal muscle tissue.

**Clinical studies and perspectives**

The clinical outcomes of vitamin D supplementation for skeletal muscle physiology have recently received increased attention (the last decade) and could result in a better understanding of the mechanisms of vitamin D effects when analysed from the perspective of the genomic pathways induced by the hormone. A vitamin D inadequacy or deficiency induces muscle fibre atrophy, slow twitch peak, may promote long periods of muscle relaxation and increased risk of chronic musculoskeletal pain\(^{(115)}\). Muscle biopsies from vitamin D-deficient adults demonstrate impairment in skeletal muscle tissue, such as fibrosis, loss of type II fibre complement and enlarged interfibrillar spaces\(^{(17,108)}\). Type II fibres are responsible for fast muscle contraction and are used predominantly to prevent falls and in power exercises and anaerobic activities\(^{(109)}\).
Many studies investigated healthy patients with baseline low serum vitamin D levels followed by supplementation with vitamin D. In these studies, vitamin D supplementation led to the activation of the VDR in skeletal muscle and consequently improved protein synthesis and muscle strength, as well as increased size and number of type II muscle fibres. A systematic review reported that vitamin D supplementation increased muscle strength (1-4 to 18-8 %) in vitamin D-insufficient athletes. In this review, the quality of controlled trials was assessed using the PEDro scale, and five RCT were identified as excellent quality and one controlled trial as good quality. Furthermore, a reduced injury incidence was reported in a group of elite ballet dancers following 4 months of 2000 IU/d of vitamin D supplementation. In a study with elite ballet dancers, significantly fewer injuries were observed after 4 months of vitamin D supplementation. Interestingly, the majority of studies investigating the effects of vitamin D in exercise performance have reported positive results in vitamin D-deficient athletes and not with adequate or supraphysiological concentrations of vitamin D. Hence, further studies are required to elucidate if supraphysiological doses of vitamin D have an ergogenic effect in vitamin D-replete athletes in different sport disciplines. Chronic long-term modifications observed in clinical studies suggest that such consequences are primarily a result of genomic actions. In disparity, genomic secondary changes in gene expression can also arise from alterations in sustained non-genomic/acute signalling.

Recent findings validated the involvement of vitamin D in the regulation of numerous skeletal and extra-skeletal cellular processes that have a direct impact on muscle function and metabolism; however, the pathways involved are incompletely understood. Vitamin D has been reported to positively influence protein synthesis, as well as increase the size of adult muscle cells and muscle mass (in animal and human studies), which could consequently result in increased muscle strength, and/or performance. Moreover, increments in skeletal muscle mass can raise the RMR and consequently the daily energy expenditure, which may benefit in the reduction of body weight in obese adults, whilst Bischoff-Ferrari et al. (147) recommended a 25(OH)D concentration of 90–100 nmol/l to enhance bone mineral density and prevent fractures in young adults. Conversely, the metabolic effects of vitamin D supplementation in non-deficient athletes remain to be clarified. In accordance with previous research, it is possible that higher serum levels of vitamin D benefit sports performance (113,130,131,156). For example, in vitro treatment with high vitamin D levels (400, 1000 nmol/l) stimulates differentiation of skeletal muscle cells and results in the maturation of myoblasts, leading to the developing of an increase in myotube fibre diameter.

Vitamin D can also affect mitochondrial metabolism, increasing O2 consumption, ATP production and modulating RMR and
energy production. Vitamin D supplementation could result in significantly better efficiency regarding energy metabolism, perhaps leading to better health outcomes, for example, preventing/reducing obesity and/or weight gain. However, insufficient data exist to confirm the effects of vitamin D on mitochondrial muscle metabolism. Observational research seems promising; however, more RCT are important to assess the effects of vitamin D supplementation in the context of muscle metabolism and function.

Conclusion

Vitamin D is associated with enhanced muscle structure and function, although the mechanisms are not yet fully understood. There is a wide range of muscle disorders related to vitamin D deficiency, and supplementation with vitamin D has mostly shown beneficial effects by counteracting the progression of diseases such as myopathy, sarcopenia, rickets and muscular dystrophy. The effects of vitamin D on proliferation and differentiation of skeletal muscle tissue in human and animal studies remain partially conflicting and need to be clarified. Finally, substantial evidence suggests that vitamin D may have the potential to modulate protein synthesis, mitochondrial metabolism as well as energy production, which is likely to make an impact on muscle strength, function and performance. Further research is required to describe the underlying mechanisms of vitamin D action on human muscle tissue, to clarify how these changes are translated into clinical effects and to define the optimal dose–effect conditions for vitamin D to obtain improvements in skeletal muscle function.

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