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 C_2C_{12}, L6 cell lines and primary human skeletal muscle cells. It is believed that 1,25-hydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), 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)$_2$D$_3$ 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 Ca^{2+} 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

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 D_2 and vitamin D_3) 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 MM) is associated with 80–90 % of total vitamin D synthesis and allows 7-dehydrocholesterol to be converted to cholecalciferol (pre-vitamin D_3)[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 D_3 is thermal isomerised to vitamin D_3, 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 in humans as it has a relatively long half-life in the circulation as it is established as a reliable marker of vitamin D status[29]. 25(OH)D is then metabolised primarily in the proximal tubule of the kidney, or other tissues such as the skeletal muscle, by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (encoded by the *CYP27B1* mitochondrial gene) to its biologically active form 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$), also known as calcitriol[27,30]. Subsequently, the resultant seco-steroid is carried by vitamin D binding protein via plasma from the kidneys to target tissues where it is able to bind to the potent transcription factor VDR. Receptor binding leads to conformational changes within the receptor that allow the VDR to interact with its heterodimeric partner, the retinoid X receptor, in the nucleus. VDR also forms homodimers that bind specific regions of DNA and activate or inhibit gene transcription[31]. The biologically active form $1,25(OH)_2D_3$ is responsible for maintaining Ca^{2+} and PO_4^{2-} homeostasis[29]. Interestingly, it has been proposed that vitamin D biological activity involves unbound or free fractions of the vitamin[32,33]. The 'free-hormone hypothesis' is considered a different pathway for cellular uptake of steroid hormones, as these molecules are highly lipophilic and therefore have the potential to quickly and passively diffuse across cell membranes[32]. It seems to be important to measure and differentiate total vitamin D (measured as 25(OH)D) and bioavailable vitamin D (i.e. free vitamin D) as important evidence suggests that vitamin D binding protein inhibits certain actions of vitamin D since the bound fraction is unavailable to act on target cells[34,35]. Powe *et al.*[36] have demonstrated in healthy adults that bone mineral density was more strongly associated with the bioavailable fraction of circulating 25(OH)D than the total levels[36]. In accordance with this result, other studies suggest that free vitamin D is a better predictor of bone mineral density especially in an ethnically diverse athletic population and also correlates better with parathyroid hormone (the marker of Ca balance associated with bone health) than total serum 25(OH)D concentration[37,38].

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 (MRF)[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 sub-population 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-sufficient 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)

Study	Species, n	VitD form, dose, time and diet	Findings and effects	Comments
Srikuea et al. (2012)[57]	C57BL/6 mice aged 12 weeks n 4	No VitD treatment Regeneration (injection of 1·2 % $BaCl_2$ to tibialis anterior) v. control group	↑ Expression of VDR and CYP27B1 gene at day 7 of regeneration v. control ($P<0.001$)	Expression of VDR in skeletal muscle during regeneration and repair
Hutton et al. (2014)[47]	Male, Ross 708 broiler chicks n 48–52 birds per group	5000 IU/kg diet with $VitD_3$ or $VitD_3$ + 25(OH)D 49 d	↑ Myogenic factor 5, ↑ density of satellite cells ($P=0.09$), ↑ total nuclear density ($P=0.05$) and ↑ muscle fibre cross-sectional area ($P=0.09$) in pectoralis major	Hypertrophic response in the pectoralis major muscle
Domingues-Faria et al. (2014)[46]	15-month-old Wistar rats n 10 per group	AIN-93M maintenance diet (1000 IU $VitD_3$/kg) or without VitD 9 months	↓ 74 % plasma 25(OH)D with VitD-deficient diet ($P<0.01$) ↓ Tibialis anterior weight in VitD-depleted rats (−25 %; $P<0.05$) In VitD-depleted group, ↓ expression of three markers of proliferation (BMP4, FGF2, PCNA; $P<0.005$)	↓ Notch pathway activity and proliferation potential
Girgis et al. (2015)[55]	C57BL/6 mice n 6–12 per group	Deletion of VDR-KO v. wild-type littermates Diet: 2·2 IU/g or VitD-deficient 3 months	Weaker grip strength: VDR-KO and VitD-deficient mice ($P<0.005$) VitD-deficient diet: ↑ myostatin, atrophy marker E3-ubiqutin ligase and MuRF1 ($P<0.005$) VDR-KO: small muscle fibre size and dysregulation of myogenic regulatory factors	Weakness advanced with age and duration of VitD deficiency
Ray et al. (2016)[54]	Female A/J mice (aged 4 weeks) n 5 per group	AIN-93-based diets with 100, 1000 or 10 000 IU $VitD_3$/kg diet 6 weeks	Maximal diaphragm force, twitch force, and fibre cross-sectional area ↓ 26, 28 and 10 % (respectively) with VitD-deficient diet ($P<0.01$)	
Oku et al. (2016)[53]	Sprague–Dawley strain male rats (aged 10 weeks) n 6	AIN-93 diet (1000 IU $VitD_3$/kg) Control, VitD restriction, high-fat diet and high-fat diet with VitD restriction 14 d	VitD restriction: ↓ volume of the femur ($P<0.001$), ↓ bone mineral density ($P<0.05$), ↓ MyoD ($P<0.05$)	Muscle mass trending towards a ↓ in VitD restriction group ($P=0.051$)

↑, 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[23]. It is important to highlight that the detection of VDR expressed in skeletal muscle cells and tissue varies according with the technique used for protein extraction and the type of primary antibody; consequently many authors have found difficulty in detecting this receptor[52].

The majority of animal studies have focused on the physiological effects of vitamin D on muscle mass and strength[53–55] 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 C_2C_{12} (Table 2), and demonstrated that treatment with vitamin D inhibits cell proliferation[23,55–59] and stimulates cell differentiation[25,58,60,61]. More recently, Braga et al.[61] have shown that treatment with $1,25(OH)_2D_3$ 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[61]. 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[48,59,60]. In addition, Ryan et al.[62] were the first group to prove a dose–response effect of the active form of vitamin D to modulate the capacity of C_2C_{12} 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[63–65]. However, in primary human skeletal muscle cells, the effects of vitamin D on proliferation and differentiation are conflicting[23,25]. Owens et al.[25] 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[25]. On the other hand, Olsson et al.[23] 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[23].

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

Study	Cell line/cell type	VitD form, dose and time	Significant findings and effects	Comments
Garcia *et al.* (2011)[56]	C_2C_{12} Myoblasts	100 nM-1,25(OH)$_2$D$_3$ 1–10 d	↓ Proliferation, ↓ IGF-1, ↑ myogenic differentiation, ↑ IGF-2, ↑ follistatin expression and ↓ myostatin	2-fold ↑ in the mean diameter of the fibres ($P<0.001$); 2·5-fold ↑ in size (width) ($P<0.001$)
Srikuea *et al.* (2012)[57]	C_2C_{12} Myoblasts, myotubes	20 nM-1,25(OH)$_2$D$_3$ or 2 μM-25(OH)$_2$D$_3$ 5 d	Inhibits cell proliferation ($P<0.001$)	↓ Myoblast number after treatment
Garcia *et al.* (2013)[58]	C_2C_{12} Myoblasts	100 nM-1,25(OH)$_2$D$_3$ 1, 3, 4, 7 or 10 d	↓ Proliferation (FGF2 and TIMP-3) ($P<0.05$) ↑ Differentiation (↑ VEGFα and FGF1, two pro-angiogenic growth factors) ($P<0.05$)	↓ Myostatin expression (negative regulator of skeletal muscle mass)
Salles *et al.* (2013)[72]	C_2C_{12} Myotubes	0, 1 or 10 nM-1,25(OH)$_2$D$_3$ 72 h	1,25(OH)$_2$D$_3$ (10 nM) + leucine and insulin, ↑ protein fractional synthesis rate (14–16 %) ($P<0.01$) ↑ mRNA expression of insulin receptor and VDR ($P<0.001$)	Akt/mTOR-dependent pathway was enhanced by 1,25(OH)$_2$D$_3$
Girgis *et al.* (2014)[48]	C_2C_{12} Myoblasts, myotubes	100 nM-1,25(OH)D$_2$, 100 nM-25(OH)D 48 h	↓ Proliferation and differentiation (↓ myostatin, myc, myogenin, myogenic factor 5 and cyclin-D; ↑ retinoblastoma protein and ATM) ($P<0.05$)	1·8-fold ↑ in cross-sectional size of individual myotubes
Irazoqui *et al.* (2014)[60]	C_2C_{12} Myoblasts	C_2C_{12} wild-type or VDR (knockdown) 1 nM-1,25(OH)$_2$D$_3$ 6, 12 and 24 h	Induces differentiation (↑ myogenin) ($P<0.05$)	p38-dependent co-localisation of VDR and cyclin D3
Owens *et al.* (2015)[25]	Human myoblasts and myotubes (biopsy)	10 or 100 nM-1,25(OH)$_2$D$_3$ 7–10 d	With 10 nM-1,25(OH)$_2$D$_3$, ↑ muscle cell migration, dynamics and ↑ myotube fusion/differentiation	Humans: improves recovery of peak torque at 48 h and 7 d post-exercise
Ryan *et al.* (2016)[24]	Human myoblasts (culture)	10 nM-1,25(OH)$_2$D$_3$ 48 h	↑ Mitochondrial oxygen consumption rate ($P=0.003$) No changes in glycolysis ($P=0.213$)	↑ Mitochondrial volume and branching
Van der Meijden *et al.* (2016)[59]	C_2C_{12} Myoblasts, myotubes	0, 400 or 1000 nM-25(OH)D or 1,25(OH)$_2$D$_3$ 24 h	↑ VDR mRNA expression and ↓ proliferation ($P<0.01$) Stimulates myosin heavy chain mRNA expression (during differentiation; $P<0.001$) ↑ Diameter of myotubes ($P<0.05$)	C_2C_{12} is able to metabolise 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$
Olsson *et al.* (2016)[23]	Human myoblasts and myotubes (biopsy)	1 or 100 nM-1,25(OH)$_2$D$_3$ 48 h	Lack of ability to convert 25(OH)D into 1,25(OH)$_2$D$_3$ Inhibits myoblast proliferation and differentiation ($P=0.014$ and $P=0.012$)	Changes in forkhead box O3 and Notch signalling pathways
Braga *et al.* (2017)[61]	Mouse skeletal muscle Satellite cells	100 nM-1,25(OH)$_2$D$_3$ 1–12 d	↑ Expression: MyoD, MYC2, myogenin, skeletal muscle fast troponin I and T, MYH1, IGF-1 and -2, FGF1 and 2, BMP4, MMP9 and follistatin ($P<0.05$)	↑ Myotube formation and expression of myostatin ($P<0.001$)

↑, Increase; ↓, decrease; 1,25(OH)$_2$D$_3$, 1,25-hydroxyvitamin D$_3$; 25(OH)D, 25-hydroxyvitamin D; Akt, protein kinase B; ATM, ataxia telangiectasia mutated; BMP4, bone morphogenetic protein family; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MMP9, matrix metallopeptidase 9; mTOR, mammalian target of rapamycin; MYC2, transcription factor; MYH1, heavy polypeptide 1; MyoD, family of myogenic regulatory factors; TIMP-3, metalloproteinase inhibitor 3; VDR, vitamin D receptor; VEGFα, vascular endothelial growth factor.

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)$_2$D$_3$) has not been confirmed to occur locally in primary skeletal muscle cells, whereas it does occur in the skeletal muscle murine cell line C_2C_{12} 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[41]. The main mechanisms that regulate protein synthesis in skeletal muscle
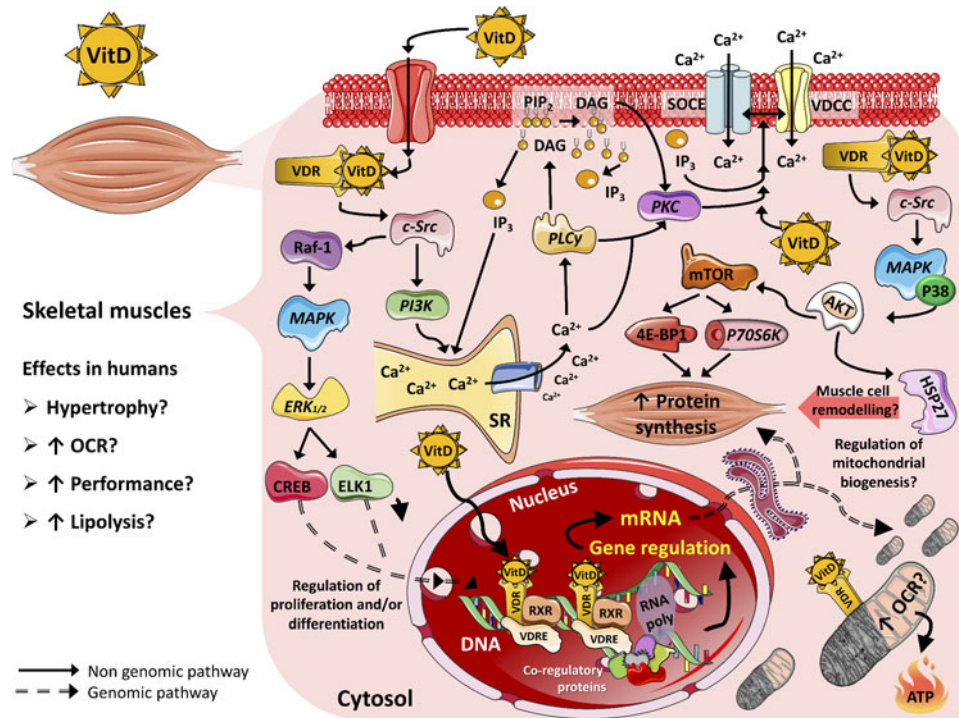
Fig. 1. Proposed mechanisms of action of vitamin D (VitD) in mammalian skeletal muscle cells. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AKT, serine–threonine kinase; Ca^{2+}, calcium ions; CREB, cellular transcription factor; c-Src, proto-oncogene c-Src; DAG, diacylglycerol; ELK1, ETS domain-containing protein; ERK1/2, extracellular signal-regulated kinases; HSP27, heat shock protein 27; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; OCR, oxygen consumption rate; P38, P38 mitogen-activated protein kinases; P70S6K, ribosomal protein S6 kinase β-1; PI3K, phosphoinositide-3 kinase; PIP2, phosphatidylinositol biphosphate; PKC, protein kinase C; PLCγ, phospholipase Cγ; Raf-1, proto-oncogene serine/threonine-protein kinase (also known as c-RAF); RNA poly, RNA polymerase; RXR, retinoid X receptor; SOCE, store-operated calcium entry; SR, sarcoplasmic reticulum; VDCC, L-type voltage-dependent calcium channel; VDR, vitamin D receptor; VDRE, vitamin D response elements.

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 β (TGF-β) 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-β signalling pathway that initially is inhibited by follistatin (activin-binding protein essential for muscle fibre formation and growth)[69,70]. Follistatin subsequently binds to multiple extracellular ligands including myostatin which modifies the transcription of Smad target genes, consequently stimulating Akt/mTOR signalling that potentiates protein synthesis[69].

The first *in vivo* 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 [³H]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 nM-1,25(OH)₂D₃ 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 3β (GSK3β), 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 C_2C_{12} 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[75].

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 $1,25(OH)_2D_3$, with a significant 2-fold increase in the mean diameter of C_2C_{12} 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 C_2C_{12} fibre diameter. Despite previous evidence of the impact of vitamin D on metabolic signalling pathways and phenotype in skeletal muscle cell lines, 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

Adequate mitochondrial function is essential for cellular homeostasis, especially in high energy-demanding skeletal muscle cells, as mitochondria are the main organelles responsible for generating energy in the form of ATP[79]. Typically, the effects of vitamin D are intermediated 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[30]. 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[26].

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 CO_2 were up-regulated by vitamin D in three immune cell types, the THP-1 monocytic 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.*[24] 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 nM-$1,25(OH)_2D_3$ 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[86]. 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 Ca^{2+} released from the sarcoplasmic reticulum during twitch responses[87]. Experiments with chick muscles deficient in vitamin D observed changes in oxidative phosphorylation and a failure of muscle mitochondria to maintain Ca^{2+}, leading to impairment of cellular metabolic homeostasis, increased reactive oxygen species and cytotoxicity due to mitochondrial dysfunction[88]. 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.

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 D_3 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[11]. 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 $1,25(OH)_2D_3$ regulates proteins that effect contractility and cell 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-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 $1,25(OH)_2D_3$ 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[31]. $1,25(OH)_2D_3$ 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 caveolae 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 Cy 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* $1,25(OH)_2D$ 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[15]. 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[75,110–112]. A systematic review reported that vitamin D supplementation increased muscle strength (1·4 to 18·8 %) in vitamin D-insufficient athletes[21]. 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[113]. In a study with elite ballet dancers, significantly fewer injuries were observed after 4 months of vitamin D supplementation[113]. 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 individuals. Three meta-analyses of RCT[114–116] reported a positive effect of vitamin D supplementation on muscle function and strength in healthy adults, while five cohort studies identified a link between serum 25(OH)D concentration and muscle strength and function[117–121]. In older adults, a recent meta-analysis found that vitamin D supplementation significantly improved muscle strength of the lower limb and femoral neck bone mineral density[122]. Moreover, Chanet *et al.*[123] have proved in healthy older men that supplementing breakfast with vitamin D and leucine enhances the postprandial muscle protein synthesis and muscle mass[123]. Higher muscle mass is associated with higher muscle strength, which is an important variable to prevent sarcopenia, falls and muscle disorders, as well as being associated with enhancement of performance in athletes. Valuable nutritional interventions combining vitamin D and amino acid supplementation could positively support muscle fibre protein synthesis in specific conditions, for example sarcopenia, where vitamin D and amino acid responses are deficient[124]. Vitamin D status is also a regulator of intestinal Ca absorption and, in turn, bone mineralisation[125]. In this context,

several reviews have concluded that there is a positive outcome for supplementation of Ca plus vitamin D for fracture risk reduction, osteoporosis and/or falls[6,126–128]. In contrast, recent meta-analyses have not been consistent with these outcomes, though many variables need to be considered when comparing studies, for example, the parameters analysed, age of the population, if participants live in residential institutions, dose and cut points of vitamin D, ethnicity, period of the intervention, instruments to assess Ca and vitamin D intake and sunlight exposure, and many others[129]. Another important variable to consider in supplementation studies is if the participant is classified as sufficient or deficient in vitamin D since the baseline period, as many of the biological effects of vitamin D are evident when status of vitamin D is corrected from deficient to normal levels[113,122,130–137].

Nutrition strategies to complement the adaptive response to exercise have been well investigated. The effects of supplementation of vitamin D on exercise and muscle health have recently been reported in soccer and rugby players, elite ballet dancers, and in active adult males that were vitamin D insufficient at the baseline[113,130–136]. Four studies[113,130,131,136] have found that supplementation of vitamin D significantly increased strength, power and exercise performance even with a small sample size, while three studies reported no significant effect of vitamin D on any physical parameter[132–134]. Further, a reduced injury incidence was reported in a group of elite ballet dancers following 4 months of 2000 IU/d of vitamin D supplementation[113]. Additionally, in a RCT, Owens *et al.*[25] showed that elevating serum 25(OH)D concentrations to >75 nmol/l of 25(OH)D after supplementation with vitamin D_3 at 4000 IU/d had a positive effect on the recovery of force following a protocol of damaging eccentric exercise[25]. In summary, evidence supports a role for vitamin D in muscle remodelling and function; however, further work is needed to clarify under what specific conditions vitamin D can be beneficial.

An important limitation related to the effect of vitamin D in muscle strength is the lack of agreement on the optimal range in blood. While plasma values of >50 nmol/l of 25(OH)D have been demonstrated to improve physiological function and assist with disease prevention, it is believed that different tissues have distinct responses to the levels of vitamin D. Hence, it is possible that the optimal 25(OH)D concentration for skeletal muscle metabolism is different from the optimal concentration for other organs/tissues[138,139–145]. For example, Heaney & Holick[146] proposed that a higher (120–225 nmol/l) serum total 25(OH)D concentration may be required for optimal skeletal muscle physiology in 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[113,130,131,136]. For example, *in vitro* treatment with high vitamin D levels (400, 1000 nM) stimulates differentiation of skeletal muscle cells and results in the maturation of myoblasts, leading to the developing of an increase in myotube fibre diameter[59].

Vitamin D can also affect mitochondrial metabolism, increasing O_2 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.

Acknowledgements

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