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The Emerging Biomolecular Role of Vitamin D in Skeletal Muscle

Rachele M. Pojednic[1] and **Lisa Ceglia**[2,3]

[1]Nutrition, Exercise Physiology and Sarcopenia Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA

[2]Division of Endocrinology, Diabetes and Metabolism, Tufts Medical Center, Boston, MA

[3]Bone Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA

Abstract

In this review, we summarize current evidence for a direct effect of vitamin D on skeletal muscle. A number of studies identify the receptor for 1,25-dihydroxyvitamin-D_3 (vitamin D receptor (VDR)) and the enzyme CYP27B1 (1-α-hydroxylase) in muscle. We hypothesize that vitamin D acts on myocytes via the VDR, and we examine proposed effects on myocyte proliferation, differentiation, growth, and inflammation.

Keywords

skeletal muscle; vitamin D; vitamin D receptor; aging; 25-hydroxyvitamin D

INTRODUCTION

Several observational studies suggest that low vitamin D status, particularly in older adults, has been associated with reduced muscle mass, strength and performance, and an increased risk of falls (6,46). Furthermore, a number of intervention studies have shown that supplementation with vitamin D increases appendicular muscle strength and performance and reduces the risk of falls mostly in older individuals with a low baseline vitamin D status (8,34). Although studies have been less consistent in younger populations, studies on vitamin D supplementation in younger vitamin D-deficient adults has suggested muscle-related benefits (38,45).

The mechanism by which vitamin D exerts these beneficial effects in skeletal muscle has not been established definitively but has been under extensive investigation in the last several years. The actions of vitamin D on skeletal muscle may, in part, be indirect by way of vitamin D's effect on calcium and phosphate homeostasis possibly via mechanisms mediated by intestinal absorption. A study in vitamin D-deficient rats found muscle

Address for correspondence: Rachele M. Pojednic, M.S., Ed.M., Nutrition, Exercise Physiology and Sarcopenia Laboratory, Jean Mayer USDA HNRCA at Tufts University, 711 Washington St., Boston, MA 02111 (rachele.pojednic@tufts.edu).

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weakness only in animals with concomitant hypophosphatemia and reported improvements in muscle strength only in the animals that had resolution of hypophosphatemia with vitamin D supplementation (37). However, recent data suggest that the actions of vitamin D on skeletal muscle also occur via a direct mechanism — the receptor for 1,25-dihydroxyvitamin-D_3 (vitamin D receptor (VDR)). Research by Srikuea *et al.* (40) localized VDR and *CYP27B1* (1-α-hydroxylase) expression in C_2C_{12} myoblasts and adult muscle cells in rodents using various techniques. Work from our laboratory, as well as those of others, also has identified VDR in human muscle biopsies (7,13) (Pojednic, R.M., *et al.*, unpublished observations, 2013) and has shown an increase in VDR concentration and single cross-sectional muscle fiber size after vitamin D supplementation (15).

We hypothesize that vitamin D acts, at least in part, on skeletal muscle tissue through the VDR in myocytes, and it may influence several key biological pathways essential to skeletal muscle function. This review summarizes current evidence for a direct biomolecular role of vitamin D in skeletal muscle. Specifically, we will examine studies focused on the localization and role of the VDR in skeletal muscle, the recent identification and significance of *CYP27B1* (1-α-hydroxylase) in skeletal muscle, and the potential skeletal muscle biological pathways influenced by vitamin D.

VITAMIN D METABOLISM

Vitamin D is regarded traditionally as a regulator of bone metabolism through the homeostatic control of calcium and phosphate. Vitamin D_3, the prohormone, is produced in the skin after exposure to sunlight and is acquired less commonly through a limited selection of dietary sources. Bound to a vitamin D binding protein, vitamin D_3 is transported to the liver where it is hydroxylated to 25-hydroxyvitamin D_3 ($25OHD_3$), the major circulating form of vitamin D and biomarker of vitamin D status. $25OHD_3$ is further hydroxylated to the active hormone, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), primarily via activity of the enzyme, 1-α-hydroxylase, encoded by the gene *CYP27B1* in the kidney and also in several other tissues including the placenta, macrophages, and monocytes (18). Notably, a recent study was able to clone the full-length CYP27B1 mRNA transcript from C_2C_{12} myotubes and found evidence of constitutive expression of CYP27B1 protein in both the cytoplasm and mitochondria of C_2C_{12} myoblasts and the cytoplasm of C_2C_{12} myotubes (40). The report also localized increased CYP27B1 protein expression in $BaCl_2$-induced regenerating fibers of mouse tibialis anterior muscle. These data provide support for the concept that skeletal muscle is a target organ of vitamin D action (Fig. 1).

VDR

Characterization of the VDR

The biologically active $1,25(OH)_2D_3$ binds to a classic steroid receptor, also known as the VDR. As described with other steroid receptors, the VDR principally acts as a nuclear transcription factor; however, a nonnuclear receptor mediating nongenomic actions also has been described (18). Within the nucleus, the VDR heterodimerizes with the retinoid × receptor (RXR) and the VDR/RXR complex binds to the VDR response element, resulting in the expression of genes involved in several components of cellular metabolism and

function (17). The characterization and mechanism of action of the putative nonnuclear VDR have not been established definitively; however, binding of $1,25(OH)_2D_3$ to a nonnuclear VDR is proposed to initiate the formation of a second messenger or phosphorylation of intracellular proteins resulting in rapid cellular effects occurring within seconds to minutes. Some have proposed it to be a novel membrane receptor (33), whereas others have suggested a membrane-associated calcium-binding protein that functions as a calcium-specific ion channel (2). However, more recent studies have characterized the nonnuclear VDR as the nuclear VDR itself, which translocates from the nucleus to the plasma membrane within cholesterol and sphingolipid-rich caveolae microdomains that concentrate components of certain signal transduction pathways (9,12,26). Garcia *et al.* (26) also used immunofluorescent analysis of VDR protein at 1 and 4 d after $1,25(OH)_2D_3$ administration in C_2C_{12} skeletal muscle cells and showed both a reduction in cytoplasmic fluorescent staining and a corresponding increase in nuclear staining from days 1 to 4, implicating translocation of the VDR to the myonucleus.

Localization of VDR in Human Skeletal Muscle

The VDR has been isolated in several human tissues (18), including skeletal muscle (7,13,20). Costa *et al.* (20) initially identified the VDR in cloned human skeletal muscle cells. Bischoff *et al.* (7) demonstrated intramyonuclear staining for the VDR using VDR antibody 97A (Affinity BioReagents) in frozen cross sections of human skeletal muscle by means of immunohistochemistry. Yet the isolation of the VDR in skeletal muscle tissue has not been consistent across all studies. A recent report questioned the selectivity of the VDR antibody 97A because it was reacting with proteins on Western blot not related to the VDR, even in a VDR knockout mouse model (44). However, more recent studies using multiple alternate VDR antibodies lend support to the presence of VDR in skeletal myocytes (13,15,27,40) (Pojednic, R.M., *et al.*, unpublished observations, 2013). A study in older women detected VDR in frozen muscle cross sections by Western blot using multiple commercial antibodies to the VDR, including a monoclonal VDR antibody D-6 (Santa Cruz Biotechnology), which is reported to have the best specificity for VDR protein because it is not detected in muscle of VDR knockout mice and C57BL/6 mice by immunohistochemistry and Western blot (13). A study by Srikuea *et al.* (40) combined the use of Western blot, immunocytochemistry, polymerase chain reaction cloning, and DNA sequencing to validate the expression and concentration of the VDR in the C_2C_{12} mouse cell line and adult mouse skeletal muscle cells. Of note, this study found VDR protein primarily expressed in the nucleus of myoblasts and adult mouse muscle cells and in the cytoplasm of C_2C_{12} myotubes. Although this last study did not use human muscle cells, the authors used multiple analytic techniques to localize the presence of VDR in skeletal muscle cells. Most recently, Girgis and colleagues (27) demonstrated that C_2C_{12} cells express VDR, *CYP27B1* (1-α-hydroxylase), *CYP24A1*, and vitamin D binding protein at the transcript level. The study also showed increased expression of VDR mRNA after 48 h of treatment with $1,25(OH)_2D_3$ with concomitant increases in *CYP24A1*, a classic VDR target gene.

With regard to human tissue, Pojednic *et al.* (unpublished observations, 2013) used Western blot and polymerase chain reaction techniques to isolate both gene and protein expression of VDR in human primary myoblasts and human biopsied vastus lateralis muscle. In addition

to VDR localization in skeletal muscle, this study also was able to demonstrate an association between serum 25OHD and alterations in VDR expression and concentration in human muscle tissue.

EVIDENCE FOR VDR-MEDIATED VITAMIN D ACTION IN SKELETAL MUSCLE

Cell and Animal Studies

Based on data in the VDR-knockout mouse model, cell culture, and other rodent studies, it has been proposed that the effects of vitamin D on skeletal muscle may, at least in part, be mediated by the VDR (Fig. 2). The VDR-knockout mouse model, for example, has muscle fibers that are approximately 20% smaller and more variable in size than those in wild-type mice, and its muscle expresses increased levels of myogenic differentiation factors including Myf5, E2A, and myogenin compared with that in the wild type (24). In addition, the VDR-null mutant mice have lower body size and weight and impaired motor coordination compared with wild-type animals. These findings were noted in animals that were corrected for metabolic and electrolyte abnormalities, particularly in calcium and phosphate.

Recent data have reported alterations in expression and concentration of the VDR in skeletal muscle cells after administration of $1,25(OH)_2D_3$. Previous studies in classical VDR target tissues, intestine and bone (21,30), have indicated that the content of VDR in these target tissues is associated positively with the level of biological activity in response to vitamin D administration. Therefore, an increase in VDR content in skeletal muscle cells after vitamin D administration would lend support to the concept that there may be effects on muscle metabolism and/or function. Three separate laboratories independently reported that VDR mRNA expression increased in C_2C_{12} myoblasts (26,27,40). Garcia *et al.* (26) and Srikuea *et al.* (40) demonstrated a more than fivefold increase of VDR after 4 d of $1,25(OH)_2D_3$ administration compared with that in control. The increased expression was further confirmed by Western blot analyses using whole-cell culture homogenates and immunofluorescence studies under similar conditions. Two of the three studies noted similar effects on VDR expression after treatment with $25OHD_3$, suggesting a common mechanism (27,40). Srikuea *et al.* (40) also examined regenerating mouse skeletal muscle *in vivo* and found that murine regenerating muscle fibers had greater expression of VDR compared with that in nonregenerating fibers, suggesting a link between muscle cell regeneration and activation of VDR.

A recent study by Tanaka and colleagues (43) demonstrated that silencing VDR in C_2C_{12} and G8 murine cells via siRNA resulted in decreased myosin heavy-chain mRNA and protein levels, as well as decreased myogenin mRNA expression and MyoD protein concentration. These authors suggest that skeletal muscle may indeed require VDR-mediated signaling for successful myoblast differentiation into myocytes.

Human Studies

Little is known about the regulation or action of the VDR in human skeletal muscle. Aging may decrease expression of the VDR in skeletal muscle, as was suggested by a previous

report (5). A recent study in older adults (Pojednic, R.M., *et al.*, unpublished observations, 2013) found a positive association between serum 25OHD and VDR concentration in skeletal muscle tissue biopsies. Furthermore, similar to recent cell culture studies (27,40), Pojednic *et al.* (unpublished observations, 2013) found an increase in VDR mRNA in human primary myoblasts treated with $1,25(OH)_2D_3$. Finally, a randomized study in 14 older vitamin D-insufficient women found that supplementation with vitamin D_3 increased intramyonuclear VDR concentration by 30% compared with that with placebo during a 4-month period (15). Despite this recent work, studies that examine actions downstream of the VDR in human muscle are lacking.

In humans, there have been a number of studies on VDR polymorphisms, or subtle alterations in the DNA sequence of the VDR gene, and the possible impact on muscle function. Polymorphisms of the VDR have been associated with variations in muscle strength in young women (28) and elderly men (1). In addition, older Japanese men and women, with varying VDR polymorphisms, demonstrated disparate increases in functional assessment of activities of daily living after a strength training intervention (32). The mechanisms by which these polymorphisms interact with skeletal muscle are yet unclear.

POTENTIAL EFFECTS OF VITAMIN D ON BIOLOGICAL PATHWAYS IN MUSCLE

Muscle Contraction and Function

Administration of $1,25(OH)_2D_3$ in muscle cell culture regulates the expression of genes that affect cell calcium handling, which is important in muscle contraction (23,25,26). $1,25(OH)_2D_3$ is thought to modulate muscle cell calcium flux by altering the activity of calcium pumps via a calcium binding protein, D9K, located in the sarcoplasmic reticulum and sarcolemma, thereby impacting muscle contraction (48). $1,25(OH)_2D_3$ also may influence muscle cell contractility by increasing the synthesis of calmodulin, a calcium binding protein that, among several other actions, regulates muscle contraction (23).

In addition, a recent intervention study in vitamin D-deficient humans found that supplementation with vitamin D augmented maximal mitochondrial oxidative phosphorylation after exercise, suggesting improvements in skeletal muscle function (38).

Muscle Cell Proliferation

Treatment of C_2C_{12} murine skeletal muscle cells with $1,25(OH)_2D_3$ decreases cellular proliferation. Garcia *et al.* (26) reported a 75% reduction in proliferating cell nuclear antigen (PCNA) expression after a 7-d incubation with $1,25(OH)_2D_3$. PCNA is a protein expressed in the nuclei of cells during DNA synthesis and, thus, is a marker of cellular proliferation. In a similar study, Srikuea *et al.* (40) and Girgis *et al.* (27) noted a significant reduction in BrdU incorporation in C_2C_{12} skeletal myoblasts after 2 d of treatment with both $25OHD_3$ and $1,25(OH)_2D_3$. BrdU is a thymidine analog that is incorporated into the DNA of replicating cells and is another indicator of cellular proliferation. Interestingly, in C_2C_{12} cells, both $1,25(OH)_2D_3$ as well as 25OHD seem to inhibit myoblast proliferation in a similar manner (27,40).

Muscle Cell Differention/Myogenesis

$1,25(OH)_2D_3$ stimulates muscle cell differentiation through the modulation of several growth factors and inhibitors. Garcia *et al.* (26) demonstrated that addition of $1,25(OH)_2D_3$ to C_2C_{12} myoblasts enhanced myogenesis by increasing the expression of promyogenic growth factors IGF-2 and follistatin and decreasing the expression of myostatin, a negative regulator of muscle mass. Tanaka *et al.* (43) found that suppressing VDR expression by RNA interference led to decreased expression of mRNA and protein levels of a variety of myogenic factors including MyoD, myogenin, MRF4, and Myf5 in C_2C_{12} and G8 cell lines. Morphological changes in the myotube formation also were noted. Additional work in C_2C_{12} cells by Garcia *et al.* (25) revealed that the promyogenic effects of $1,25(OH)_2D_3$ may further involve effects on angiogenesis via increased expression of angiogenic growth factors VEGFα and FGF-1 and decreased angiogenic inhibitors FGF-2 and TIMP-3.

Muscle Growth

Several studies have examined the effect of vitamin D on known anabolic signaling pathways involved in skeletal muscle growth. Treatment of C_2C_{12} myotubes with $1,25(OH)_2D_3$ sensitized the Akt/mTOR-dependent pathway to the known stimulating effect of leucine and insulin, resulting in a further activation of protein synthesis (35). In rats on an alkali diet, vitamin D_3 supplementation enhanced phosphorylated Akt protein concentration, an established component of the skeletal muscle anabolic cascade (14). Lastly, $1,25(OH)_2D_3$ has been shown to upregulate Akt directly through Src, PI3K, and p38 MAPK, which stimulate myogenesis in C_2C_{12} cells (11), potentially through a VDR-dependent mechanism (10).

Notably, further support in the potential role of vitamin D in muscle growth stems from human muscle biopsy studies examining effects at the histological level by measuring changes in muscle fiber size. An uncontrolled study in 11 older osteoporotic women with profound vitamin D deficiency found an increase in Type IIa muscle fiber cross-sectional area after 3 to 6 months of treatment with 1-α-hydroxyvitamin D and calcium (39). A randomized study found that treatment of older Japanese female stroke survivors with 1000 IU of vitamin D_2 daily increased the mean Type II muscle fiber diameter by more than 90% during a 2-yr period in the nonparetic limb compared with placebo (36). In these rehabilitated women whose baseline $25OHD_3$ levels were less than 25 nmol/L, there also was a correlation between serum $25OHD_3$ level and Type II muscle fiber diameter both at baseline and after 2 yr of follow-up. Finally, a recent intervention study found a 10% increase in total (Types I and II) muscle fiber cross-sectional area after 4 months of vitamin D_3 supplementation versus placebo in older mobility-limited and vitamin D-insufficient women (15). This relationship is corroborated in C_2C_{12} cells *in vitro*, by which myotubes are increased markedly in diameter after 10-d treatment with $1,25(OH)_2$-D_3, as well as 25OHD, potentially through an inhibitory effect of $1,25(OH)_2D_3$ on myostatin (27).

Muscle Inflammation

Vitamin D status has been implicated in the regulation of inflammation in nonmuscle tissue. Vitamin D, particularly the active hormone $1,25(OH)_2D_3$, has been shown to suppress the production of several proinflammatory cytokines in serum, including interleukin 6 (IL-6)

and tumor necrosis factor-α (TNF-α) (29), as well as reduce nuclear factor-KB (NFKB) expression, binding, and transcriptional activity in human and mouse fibroblasts and dendritic cells (22,31). These actions are thought to be mediated by the VDR, which has been localized in immune cells and fibroblasts. Activation of the VDR on mouse embryonic fibroblasts seems to inhibit NFKB activation and subsequent synthesis of proinflammatory cytokines (42). Furthermore, VDR-null mutant mice have increased markers of intestinal epithelial cell inflammation both intracellularly via NFKB and in serum via IL-6, a well-known NFKB target gene (47).

Based on evidence from nonmuscle tissues and increasing knowledge with regard to skeletal muscle, vitamin D also may play a role in the regulation of pathways of intramuscular inflammation. Vitamin D status has been associated with reduced circulatory inflammatory markers and concomitant impairments in peak power in healthy adults (4). Serum 25OHD also has been implicated in skeletal muscle recovery after exercise and injury (3,16,41). In humans, vitamin D supplementation resulted in an attenuation of exercise-induced muscle weakness compared with control (3). In rats exposed to crush injury, vitamin D supplementation resulted in significant increases in muscle cell regeneration and extracellular matrix proteins and decreases in muscle cell apoptosis (41). In a randomized controlled study in rats, vitamin D influenced exercise-induced muscle damage and inflammation through the modulation of MAPK and NF-KB pathways possibly mediated by VDR (16). Specifically, this latter study found that the skeletal muscle of vitamin D-treated rats exposed to high-intensity exercise demonstrated an increase in skeletal muscle VDR with reduced expression of p38, ERK1/2, IKK, and IKB, key regulatory kinases in the inflammation signaling cascade, and subsequent reductions in proinflammatory TNF-α and IL-6 when compared with that with placebo (16).

CONCLUSIONS

A growing number of studies have suggested a beneficial effect of vitamin D on skeletal muscle strength and physical performance, particularly in older adults. Emerging evidence from both preclinical and clinical studies has begun to elucidate major molecular mechanisms by which vitamin D acts on skeletal muscle cells. Recent data localizing *CYP27B1* (1-α-hydroxylase) and VDR protein expression in skeletal muscle cells provide support for a direct action of vitamin D. In addition, VDR-knockout mouse models and VDR polymorphisms further implicate that the VDR plays a role in skeletal muscle cell development and function. As in classic vitamin D target tissues, administration of vitamin D increases *VDR* gene and protein expression in muscle, suggesting increased biological activity. Although VDR downstream signaling cascades in skeletal muscle have yet to be well characterized, recent cell culture data suggest that major biological pathways including muscle contraction, cell proliferation, cell differentiation, growth, and inflammation are altered after the administration of vitamin D.

Despite the advances in understanding of the underlying molecular actions of vitamin D in skeletal muscle, there still are many gaps in knowledge that require further investigation. As molecular and cellular mechanisms become better understood, the next step will be to

examine how these tissue-level events translate into changes in muscle mass, strength, and function.

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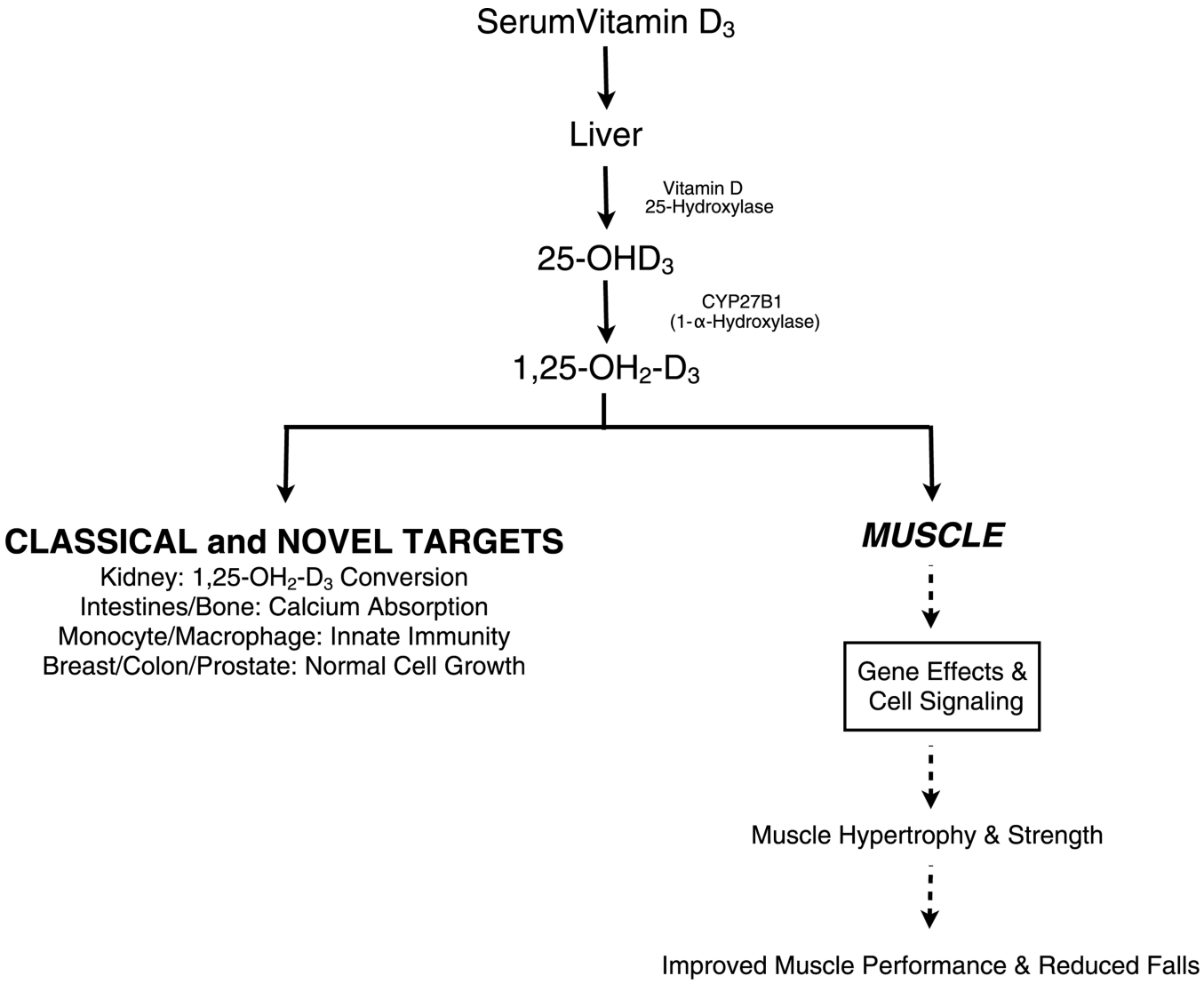

Figure 1.

Classical and novel targets for vitamin D. Vitamin D has been traditionally accepted as a mediator of calcium absorption in the intestine, resulting in subsequent effects on bone. More recent actions have been reported in a variety of other tissues, most recently in muscle.

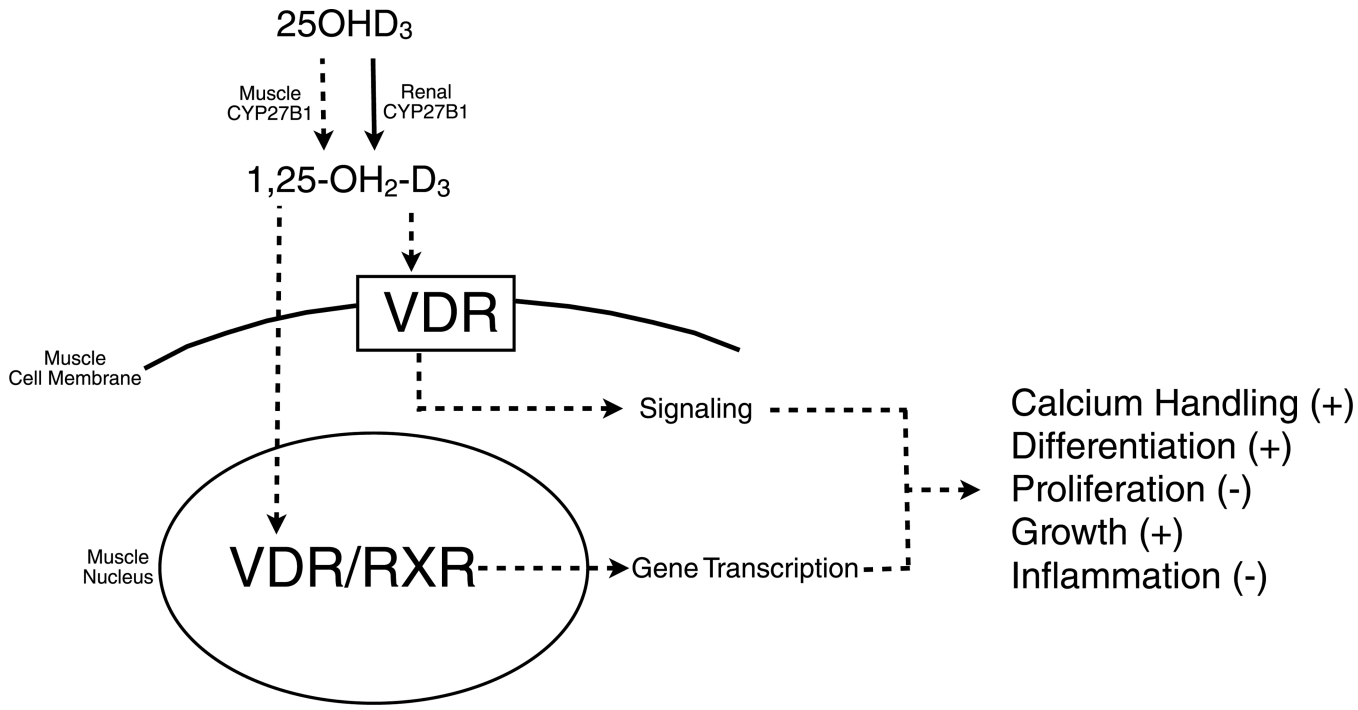

Figure 2.

Proposed hypothesis model for a direct effect of vitamin D on skeletal muscle. Circulating and locally converted $1,25(OH)_2D_3$ have been proposed to act on skeletal muscle through the VDR (*dotted line*). The VDR is located both within the nucleus, which results in genomic actions, and outside of the nucleus, which may cause acute nongenomic signaling events. Current data suggest that the biological pathways affected by $1,25\text{-}OH_2\text{-}D_3$ in skeletal muscle include calcium handling/contraction, cellular differentiation and proliferation, growth pathways, and inflammation.