Effects of intramuscular administration of 1α,25(OH)2D3 during skeletal muscle regeneration on regenerative capacity, muscular fibrosis, and angiogenesis

Ratchakrit Srikuea1 and Muthita Hirunsai2
1Department of Physiology, Faculty of Science, Mahidol University, Bangkok; and 2Department of Biopharmacy, Faculty of Pharmacy, Sirirakarinwirot University, Nakhon Nayok, Thailand

Submitted 2 December 2015; accepted in final form 27 March 2016

Srikuea R, Hirunsai M. Effects of intramuscular administration of 1α,25(OH)2D3 during skeletal muscle regeneration on regenerative capacity, muscular fibrosis, and angiogenesis. J Appl Physiol 120: 1381–1393, 2016. First published March 31, 2016; doi:10.1152/japplphysiol.01018.2015—The recent discovery of the vitamin D receptor (VDR) in regenerating muscle raises the question regarding the action of vitamin D3 on skeletal muscle regeneration. To investigate the action of vitamin D3 on this process, the tibialis anterior muscle of male C57BL/6 mice (10 wk of age) was injected with 1.2% BaCl2 to induce extensive muscle injury. The bioactive form of vitamin D3 [1α,25(OH)2D3] was administered daily via intramuscular injections during the regenerative phase (days 4–7 postinjury). Physiological and supraphysiologics doses of 1α,25(OH)2D3 relative to 1 μg/kg muscle weight and mouse body weight were investigated. Muscle samples were collected on day 8 postinjury to examine proteins related to vitamin D3 metabolism (VDR, CYP24A1, and CYP27B1), satellite cell differentiation and regenerative muscle fiber formation [myogenin and embryonic myosin heavy chain (EbMHC)], protein synthesis signaling (Akt, p70 S6K1, 4E-BP1, and myostatin), fiber-type composition (fast and slow MHCs), fibrous formation (vimentin), and angiogenesis (CD31). Administration of 1α,25(OH)2D3 at physiological and supraphysiologics doses enhanced VDR expression in regenerative muscle. Moreover, CYP24A1 and vimentin expression was increased, accompanying decreased myogenin and EbMHC expression at the supraphysiologics dose. However, there was no change in CYP27B1, Akt, p70 S6K1, 4E-BP1, myostatin, fast and slow MHCs, or CD31 expression at any dose investigated. Taken together, administration of 1α,25(OH)2D3 at a supraphysiologics dose decreased satellite cell differentiation, delayed regenerative muscle fiber formation, and increased muscular fibrosis. However, protein synthesis signaling, fiber-type composition, and angiogenesis were not affected by either 1α,25(OH)2D3 administration at a physiological or supraphysiologics dose.

vitamin D3; intramuscular injection; muscle regeneration; muscular fibrosis; angiogenesis

NEW & NOTEWORTHY

The presence of the vitamin D receptor (VDR) and vitamin D3 metabolizing enzymes (CYP24A1 and CYP27B1) in regenerating muscle suggests local metabolism of vitamin D3 in this extrarenal tissue. Although intramuscular administration of the bioactive form of vitamin D3 [1α,25(OH)2D3] at a physiological dose had no significant impact on skeletal muscle regeneration, a supraphysiologics administration into regenerating muscle resulted in impaired satellite cell differentiation, delayed regenerating muscle fiber formation, and increased muscular fibrosis.

VITAMIN D3 (CHOLECALCIFEROL) is a prohormone that is essential for calcium homeostasis (12). Furthermore, a broad range of physiological noncalcemic functions of vitamin D3 have been reported, e.g., controlling cell proliferation, differentiation, and apoptosis (23). The metabolism of vitamin D3 from precursor to its bioactive form to initiate its action on the target tissue requires multiple steps. Briefly, synthesis of vitamin D3 occurs in the skin after the vitamin D3 precursor (7-dehydrocholesterol) is exposed to sunlight to form cholecalciferol. Cholecalciferol is hydroxylated in the liver to form 25(OH)D3 (calcidiol). Calcidiol is further hydroxylated via 1-α hydroxylase, a cytochrome P450 containing hydroxylase encoded by the cyp27b1 gene, in the kidney to produce the bioactive form of vitamin D3, 1α,25(OH)2D3 (calcitriol). The concentration of 1α,25(OH)2D3 is regulated by the action of the vitamin D3 catalyzing enzyme, which is encoded by the cyp24a1 gene, to convert 1α,25(OH)2D3 to the inactive metabolite calcitriolic acid (20).

The action of 1α,25(OH)2D3 requires binding to the vitamin D receptor (VDR) to initiate downstream cascades and modulate gene transcription. VDR is localized as a nuclear receptor and acts as a transcription factor that recognizes cognate vitamin D response elements in more than 3,000 target genes in the mouse genome (28). In addition to calcemic regulatory genes, myogenic regulatory genes (Myf5, MyoD, myogenin, and myostatin) (4, 6, 8) and muscle phenotypic genes [myosin heavy chain (MHC) isoform] (4, 6, 16) were modulated in responses to 1α,25(OH)2D3 treatment. The action of 1α,25(OH)2D3 on skeletal muscle cells and tissue was supported by the presence of VDR protein in a mouse muscle cell line (C2C12) (6, 8, 26), rodent skeletal muscle (4, 13, 26, 27), human primary myoblasts (17, 19), and human skeletal muscle (1, 2, 18). Moreover, two major enzymes regulating vitamin D3 metabolism, CYP27B1 and CYP24A1, were also expressed in C2C12 cells and mouse primary myotubes (9). Taken together, expression of the VDR and vitamin D3 metabolizing enzymes in skeletal muscle suggests the possible local regulation of vitamin D3 on this extrarenal tissue.

The presence of VDR protein in regenerative muscle, as demonstrated by immunohistochemistry (26) and Western blot analysis (27), raised a major question regarding the potential role of 1α,25(OH)2D3 on the regulation of skeletal muscle regenerative capacity. Skeletal muscle regeneration is a self-repairing process following muscle injury. This process involves the activity of resident skeletal muscle stem cells (satellite cells) that are located between the basement membrane and basal lamina of muscle fibers (14). Satellite cells are unipotent and can differentiate to form muscle fibers in addi-

Address for reprint requests and other correspondence: R. Srikuea, Dept. of Physiology, Faculty of Science, Mahidol Univ., Bangkok 10400, Thailand (e-mail: ratchakrit.sri@mahidol.ac.th).

http://www.jappl.org 8750-7587/16 Copyright © 2016 the American Physiological Society 1381
tion to being self-renewing (21). Under normal condition, satellite cells are quiescent but can become active, proliferating and differentiating after injury to repair damaged muscle fibers or generate new muscle fibers (10). After activation, satellite cells express transcription factors that can be used to identify the stages of their activity, i.e., MyoD, Myf5, Myf6 (activation, proliferation, and differentiation), and myogenin (differentiation) (25). Currently, the satellite cell population can be determined by the expression of the specific transcription factor Pax7 (24). The central nuclei derived from satellite cell fusion in regenerating muscle fibers are a hallmark of muscle regeneration. These newly formed muscle fibers after muscle injury can be characterized by the expression of embryonic myosin heavy chain (EbMHC) (15).

The efficiency of the regenerative process requires a sufficient myoblast-derived satellite cell number, effectiveness of myoblast fusion and differentiation, and a rapid increase in the rate of protein synthesis. The suppressive effects of 1α,25(OH)2D3 on myogenic cells have been reported in various studies on myoblast proliferation (6, 8, 26) and myotube formation (8). Although its effect leads to a decrease in myotube number, an increase in myotube size in vitro has been reported (8). In addition, treatment with 1α,25(OH)2D3 in combination with insulin and leucine increased the phosphorylation of the Akt/mTOR pathway, suggesting an anabolic effect in skeletal muscle cells (22). An impaired muscle fiber regenerative response occurs when mTOR is inhibited after muscle injury (7); however, the direct effect of 1α,25(OH)2D3 on Akt/mTOR pathway activation in regenerative muscle has not been investigated. To our knowledge, only one investigation reported an effect of vitamin D3 on muscle regeneration (7); however, the direct effect of 1α,25(OH)2D3 dose on skeletal muscle regeneration using a crush injury model (27). Administration of activated 7-dehydrocholesterol via subcutaneous administration to the rats immediately after contusion-induced muscle injury leads to partial restoration of muscle function. However, VDR protein expression and satellite cell activity did not change in response to activated 7-dehydrocholesterol administration. Furthermore, an investigation on the direct action of the bioactive form of vitamin D3, 1α,25(OH)2D3, on skeletal muscle regeneration has not been investigated.

Connective tissue formation and angiogenesis act as contributing factors to determine skeletal muscle regenerative capacity. Increased fibroblast proliferation and collagen production after activated 7-dehydrocholesterol administration has been demonstrated (27). However, a single dose of activated 7-dehydrocholesterol immediately after muscle injury leads to an increased serum 25(OH)D3 that suppressed 1α,25(OH)2D3 serum level. This leaves the direct action of 1α,25(OH)2D3 on regulation of connective tissue formation/muscular fibrosis in regenerative muscle still unanswered. Moreover, the effect of 1α,25(OH)2D3 on proangiogenic growth factors and angiogenic inhibition was recently discovered (5). Treatment of 1α,25(OH)2D3 to C2C12 myoblasts increased vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor-1 (FGF-1) while suppressing FGF-2 and tissue inhibitor of metalloproteinase-3 (TIMP-3). However, this study was conducted in vitro, demonstrating a need to clarify 1α,25(OH)2D3 action on angiogenesis during skeletal muscle regeneration in vivo.

Taken together, the aim of this study was to investigate action of the bioactive form of vitamin D3 [1α,25(OH)2D3] on the skeletal muscle regenerative process. Intramuscular injection of 1α,25(OH)2D3 at physiological and supraphysiological doses was investigated to determine the direct action of 1α,25(OH)2D3 on muscle regenerative capacity, muscular fibrosis, and angiogenesis during skeletal muscle regeneration. Local administration of 1α,25(OH)2D3 was applied in this study to bypass the vitamin D endocrine system that involves finely regulated control at the kidney, which may alter the concentration of administered 1α,25(OH)2D3. In addition, the expression of VDR and vitamin D3 metabolizing enzymes (CYP24A1 and CYP27B1) in regenerative muscle after intramuscular injection of 1α,25(OH)2D3 was examined to clarify the presence of local metabolism of 1α,25(OH)2D3 during skeletal muscle regeneration.

**MATERIALS AND METHODS**

**Animals**

Adult male C57BL/6 mice were obtained from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand. All animal procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals as approved by the Ethics Committee on the Use of Experimental Animals, Faculty of Science, Mahidol University, Thailand (Protocol No. MUSC56-005-267).

**BaCl2-induced Muscle Injury**

C57BL/6 mice (10 wk of age) were anesthetized by inhalation with isoflurane gas prior to injury induction. Left and right tibialis anterior (TA) muscles were injected intramuscularly with 50 μL of 1.2% BaCl2 dissolved in sterile PBS to induce extensive muscle injury.

**Intramuscular 1α,25(OH)2D3 Administration**

Mice were randomly assigned to one of three groups (n = 6/group): 1) control group, 2) injury group + 1 physiological dose of 1α,25(OH)2D3 [dose: 1 μg/kg relative to TA muscle wet weight (MW)], and 3) injury group + a supraphysiological dose of 1α,25(OH)2D3 [dose: 1 μg/kg relative to mouse body weight (BW)]. Physiological and supraphysiological doses of 1α,25(OH)2D3 were selected in this study to determine the differential effects of a 1α,25(OH)2D3 dose on skeletal muscle regenerative capacity, muscular fibrosis, and angiogenesis. 1α,25(OH)2D3 (71820, Cayman Chemical) was administered daily via intramuscular injection to the left injured TA muscle on days 4–7 postinjury. The right injured TA served as contralateral injured muscle and was administered a vehicle treatment. The administration period of 1α,25(OH)2D3 represents the phase of muscle regeneration that involves satellite cell differentiation, rapid increases in protein synthesis, regenerative muscle fiber formation, extracellular matrix remodeling, and revascularization.

**Muscle Wet Weight/Body Weight Ratio**

On day 8 postinjury, injured TA muscles from either vehicle- or 1α,25(OH)2D3-administered groups were dissected and weighed with a digital weight scale to within ± 0.001 mg (MS204S, Mettler Toledo). Immediately after dissection, excess fluid was removed with filter paper. TA muscle wet weight (mg) was measured, normalized to mouse body weight (g), and recorded as muscle wet weight to body weight ratio.

**Western Blot Analysis**

Muscle samples were homogenized in ice-cold homogenizing buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with protease inhibitor cocktail (Sigma, P8340) (1:100), and phosphatase inhibitor cocktail (Calbiochem,
Muscle homogenates were centrifuged at 1,500 g for 10 min at 4°C to separate cytosolic and myofibrillar fractions. The cytosolic fraction was further centrifuged at 10,000 g for 10 min at 4°C, and the supernatant portion was collected. To remove debris in the myofibrillar fraction, pellets were suspended in the same ice-cold homogenizing buffer and centrifuged at 1,500 g for 10 min × 3 (4°C). Thereafter, the insoluble pellet was resuspended with 0.5 M NaOH in 50 mM Tris-HCl, pH 7.5. Protein concentrations were determined by BCA assay in triplicate measurements. Under a reducing condition, 40 μg of cytosolic protein and 10 μg myofibrillar protein fractions containing sample buffer solution were denatured by heating at 60°C for 10 min. The protein samples were loaded into SDS-polyacrylamide gel (5% stacking and 8–10% separating gels) and then transferred to a PVDF membrane (IPVH-00010, Millipore). The membrane was blocked with 5% nonfat milk (1706404XTU, Bio-Rad) and probed with primary antibodies. The cytosolic proteins were incubated overnight with the following primary antibodies: VDR (D-6) (1:200, SC-13133, Santa Cruz Biotechnology), CYP24A1 (1:200, SC-66851, Santa Cruz Biotechnology), CYP27B1 (1:200, SC-67261, Santa Cruz Biotechnology), myogenin (1:200, SC-12732, Santa Cruz Biotechnology), myostatin (1:200, SC-393335, Santa Cruz Biotechnology), vimentin (1:200, SC-32322, Santa Cruz Biotechnology), CD31 (1:500, Ab28364, Abcam), Phospho-P70 S6K1 Thr389 (1:1,000, 9206, Cell Signaling), phospho-4E-BP1 (1:1,000, 9451, Cell Signaling), 4E-BP1 (1:1,000, 9644, Cell Signaling), phospho-Akt Ser473 (1:1,000, 4060, Cell Signaling), and Akt Ser473 (1:2,000, 2967, Cell Signaling). However, the myofibrillar proteins were incubated with the following primary antibodies: fast MHC (1:2,000, ab91506, Abcam), slow MHC (1:2,000, ab11083, Abcam), and EbMHC (1:500, SC-53091, Santa Cruz Biotechnology). Particularly, fast MHC and slow MHC antibodies also applied with cytosolic protein fraction to determine soluble pool of MHCs. Thereafter, the membrane was incubated with appropriate horseradish peroxidase conjugated secondary antibodies. Protein bands were visualized with chemiluminescence horseradish peroxidase detection reagent (WBLUR0100, Millipore) and exposed to CL-XPosure film (PIE34090, Thermo Scientific). Band density was normalized with amount of loading protein as determined by Ponceau S staining.

Histological Analysis

The frozen muscle samples were sectioned at 10-μm thickness with cryostat (Model CM1850, Leica). To determine cross-sectional area (CSA) of regenerative muscle fibers, the muscle sections were stained with hematoxylin and eosin to visualize the presence of central nucleated regenerative muscle fibers that represents the hallmark of muscle regeneration. Representative images were taken at ×200 magnification with Olympus microscope BX53 (Olympus, Japan). Six images were randomly captured with cellSens Dimension version 1.81 software (Olympus, Japan) for quantitative analysis. Regenerative muscle fiber CSA was quantified by using ImageJ software version 1.44p.

Immunohistochemical Analysis

The serial sections at 10-μm thickness were used to investigate the changes of VDR, CYP24A1, CYP27B1, ATP synthase (complex V) subunit α (OxPhos), Pax7, myogenin, and vimentin-stained sections were prefixed with 4% paraformaldehyde (PFA) for 10 min. In contrast, CD31-stained sections were prefixed with ice-cold acetone for 10 min while fast MHC, slow MHC, and EbMHC-stained sections were unfixed at this step. For nuclear localization proteins (VDR, Pax7, and myogenin), the sections were permeabilized with 0.5% Triton X-100 for 5 min. Then the sections were blocked with mouse IgG blocking reagent (MKB-2213, Vector Labs) for 1 h followed with 10% normal goat serum (PCN5000, Invitrogen) for 1 h. Primary antibodies were incubated overnight at 4°C, and dilution used for immunohistochemical analysis were listed as follows: mouse monoclonal VDR (D-6) (1:50), rabbit polyclonal VDR (H-81) (1:50), rabbit polyclonal CYP24A1 (1:50), rabbit polyclonal CYP27B1 (1:50), mouse monoclonal ATP synthase (complex V) subunit α (OxPhos) (1:200), mouse monoclonal myogenin (1:50), rabbit polyclonal fast MHC (1:1,000), mouse monoclonal slow MHC (1:2,000), mouse monoclonal EbMHC (1:100), mouse monoclonal vimentin (1:100), and rabbit polyclonal CD31 (1:100). Rabbit polyclonal laminin antibody (1:400, L9393, Sigma) was used to visualize regenerative muscle fiber structure. Thereafter, the sections were incubated with goat anti-mouse Alexa 568 (1:500, A-11004, Invitrogen) and goat antirabbit Alexa 488 (1:500, A-11008, Invitrogen) for 1 h in the dark. Fast MHC, slow MHC, and EbMHC-stained sections were postfixed with 4% PFA for 10 min. All stained sections were mounted with anti-fade containing DAPI (H-1200, Vector Labs). Representative images were taken at ×100, ×200, or ×400 magnification with Olympus microscope BX53 (Olympus, Japan). Six to eight images were randomly captured at ×200 magnification with cellSens Dimension version 1.81 software (Olympus, Japan) for quantitative analysis. Myogenin-positive nuclei counting and quantitative expression analysis of EbMHC, vimentin, and CD31 protein were performed with ImageJ software version 1.44p.

Cell Culture

The C2C12 mouse muscle cell line (source: CRL-1772, American Type Culture Collection) was used in this study. This cell line has been widely used to test the myogenic differentiation capacity that represents satellite cell differentiation during skeletal muscle regeneration in vivo. Briefly, C2C12 myoblasts were cultured with growth medium (DMEM + 10% fetal bovine serum) in a 6-well plate at a starting density 5 × 10⁴ cells/well. When cell density reached ~70% confluency, growth medium was replaced with differentiation medium (DMEM + 2% horse serum) to activate cell fusion and differentiation to form myotubes. At this step, differentiation medium was supplemented with either vehicle or 1α,25(OH)2D3 at final concentrations of 0.1, 1, and 10 nM and refreshed daily for 4 days to test the direct effect of 1α,25(OH)2D3 on myogenic cell differentiation and myotube formation. The changes of myogenin and EbMHC protein expression were investigated with immunocytochemistry. In another set of experiments, C2C12 myoblasts were seeded at density 2.5 × 10⁴ cells/well and cultured with growth medium in a 12-well plate for 24 h. Thereafter, cells were treated with 1α,25(OH)2D3 at final concentration 10 nM for 24 h to determine VDR and CYP24A1 protein expression and localization. The mitochondrial compartment of the treated C2C12 myoblasts was localized with ATP synthase (complex V) subunit α (OxPhos).

Immunocytochemistry

Briefly, cells were fixed with 4% PFA for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. The permeabilization step was performed specifically for nuclear localization protein staining (VDR and myogenin proteins). Cells were blocked with 10% normal goat serum for 30 min and incubated with primary antibodies as follows: VDR (D-6) (1:100), CYP24A1 (1:100), ATP synthase (complex V) subunit α (OxPhos) (1:200), myogenin (1:100), and EbMHC (1:200) for 1 h. Cells were incubated simultaneously with goat anti-
Alexa 568 (1:500) and goat antirabbit Alexa 488 (1:500) for 1 h in the dark. Nuclei were counterstained with DAPI (1:1,000, Invitrogen) for 5 min, and a temporary mounting medium (20% PBS plus 80% glycerol) was applied. Representative images were captured at either ×100 or ×200 magnification with an Olympus microscope IX83 (Olympus, Japan). Images for quantitative analysis were randomly captured at ×100 magnification. The quantitative analysis of myogenin-positive nuclei and EbMHC protein expression was performed with cellSens Dimension version 1.81 software.

Statistical Analysis

Data are presented as means ± SE. Normal distribution and homogeneity of variance were determined by a Shapiro-Wilk test and Levene’s test, respectively. Significant differences among groups were analyzed with either one-way ANOVA with Tukey’s post hoc test or Kruskal-Wallis with Dunn’s multiple comparison test where appropriate. Data were analyzed with SPSS version 18.0.0, and the level of statistical significance was set with α level of \( P < 0.05 \).

RESULTS

Effect of Consecutive Intramuscular Administrations on Muscle Wet Weight to Body Weight Ratio

Muscle wet weight (MW) to body weight (BW) ratio was determined to evaluate whether consecutive intramuscular administration of either a vehicle or 1α,25(OH)\(_2\)D\(_3\) at a physiological dose (1 μg/kg relative to TA muscle) and a supraphysiological dose (1 μg/kg relative to mouse BW) during the regenerative phase (days 4–7 postinjury) had an effect on muscle edema that may impact skeletal muscle regenerative capacity. The experimental diagram for consecutive intramuscular administration of either vehicle or 1α,25(OH)\(_2\)D\(_3\) is illustrated in Fig. 1A. The results demonstrated that the TA muscle wet weight to mouse body weight ratio significantly decreased during skeletal muscle regeneration in the vehicle-administered dose (1.54 ± 0.05 mg/g BW), 1α,25(OH)\(_2\)D\(_3\) administered at the physiological (MW) dose (1.58 ± 0.02 mg/g BW), and the supraphysiologic (BW) dose (1.57 ± 0.08 mg/g BW) compared with control group (1.81 ± 0.02 mg/g BW) \( P < 0.05 \) (Fig. 1B). Irrespective of treatment [vehicle- or 1α,25(OH)\(_2\)D\(_3\)-administered group], the muscle wet weight to body weight ratios were not significant different. These data suggest that muscle edema was not apparent after consecutive intramuscular administration of either vehicle or 1α,25(OH)\(_2\)D\(_3\) at both physiological (MW) and supraphysiological (BW) doses during skeletal muscle regeneration.

VDR Protein Was Expressed in Regenerative Muscle Fibers and Satellite Cells

To determine if skeletal muscle regenerative capacity could be modulated by 1α,25(OH)\(_2\)D\(_3\), VDR protein expression in regenerative muscle fibers and satellite cells was verified using the highly sensitive VDR antibody VDR (D-6) (31). First, we demonstrated that regenerative muscle fibers expressed VDR protein as determined by immunostaining with the mouse VDR (D-6) antibody (Fig. 2A). Since regenerative muscle fiber formation is dependent on the function of satellite cells, we further determined whether satellite cells are a target of 1α,25(OH)\(_2\)D\(_3\) in regenerative muscle. Counterstaining with both mouse Pax7 antibody and a rabbit VDR (H-81) antibody that was previously reported to detect VDR protein expression in regenerative muscle fibers (26) was performed (Fig. 2B). The results revealed that in addition to regenerating muscle fibers, satellite cells also expressed VDR protein. Furthermore, the specificity of VDR staining in regenerative muscle with rabbit VDR (H-81) antibody was verified with mouse VDR (D-6) antibody (Fig. 2C).

Intramuscular Administration of 1α,25(OH)\(_2\)D\(_3\) into Regenerative Muscle Increased VDR Protein Expression

Since VDR protein was expressed in regenerating muscle fibers and satellite cells, this suggests a possible role of 1α,25(OH)\(_2\)D\(_3\) in regulating muscle regenerative capacity. Therefore, intramuscular administration of 1α,25(OH)\(_2\)D\(_3\) during skeletal muscle regeneration was investigated. Physiological (MW) and supraphysiological (BW) doses of 1α,25(OH)\(_2\)D\(_3\) were administered for 4 consecutive days during days 4–7 postinjury (regenerative phase). Western blot analysis using VDR (D-6) antibody revealed that VDR protein was expressed at a barely detectable level in control compared with regenerative muscle (Fig. 2D). VDR protein expression was significantly increased 5.6 ± 1.7-fold during muscle regeneration in the vehicle-administered group \( P < 0.05 \) and further increased in response to 1α,25(OH)\(_2\)D\(_3\) administration at physiological (MW) dose (8.1 ± 2.2-fold) and supraphysiological (BW) dose (9.9 ± 3.1-fold) \( P < 0.01 \) compared with...
the control group (Fig. 2D; two sets of samples were illustrated). These results suggest that intramuscular administration of 1α,25(OH)2D3 during muscle regeneration enhanced VDR protein expression in regenerative muscle.

**Intramuscular Administration of 1α,25(OH)2D3 During Muscle Regeneration Increased CYP24A1 but not CYP27B1 Protein Expression**

To understand the regulation of vitamin D3 during skeletal muscle regeneration, we sought to determine the effect of intramuscular administration of 1α,25(OH)2D3 on alterations in vitamin D3 metabolizing enzymes in regenerative muscle. It is known that 1α,25(OH)2D3 concentration is regulated by two vitamin D3 regulating enzymes, CYP24A1 and CYP27B1. In general, excess 1α,25(OH)2D3 can be converted to its catabolite form 1α,24,25(OH)2D3 via the CYP24A1 enzyme in which 1α,24,25(OH)2D3 is less active and has a lower affinity for VDR binding. In contrast, CYP27B1 functions to increase 1α,25(OH)2D3 synthesis from 25(OH)D3 in both renal and extrarenal tissue. In the present study, Western blot analysis revealed that administration of 1α,25(OH)2D3 at supraphysiological (BW) dose significantly increased CYP24A1 protein expression by 1.5 ± 0.2-fold compared with the control group (P < 0.05), while no significant difference was detected at physiological (MW) dose (Fig. 3A). Immunohistochemical analysis revealed that the CYP24A1 protein was expressed in regenerative muscle and its localization was present at both regenerative muscle fibers and the extracellular matrix. Interestingly, CYP24A1 protein was colocalized with VDR protein in the central nuclei of regenerative muscle fibers (Fig. 3B). This result suggests the possible local regulation of 1α,25(OH)2D3 during skeletal muscle regeneration by the enzymatic machinery of the vitamin D3 endocrine system. The specific nuclear localization of the CYP24A1 protein in regenerative muscle fibers was further clarified in mouse C2C12 myoblasts. Results clearly demonstrate that CYP24A1 is mainly expressed in the nuclei of myoblasts which colocalized with VDR protein after 1α,25(OH)2D3 treatment (Fig. 3C, top). Furthermore, the CYP24A1 protein was expressed at low levels in the mitochondrial compartment as determined by counterstaining with ATP synthase (complex V) subunit α (OxPhos) to show mitochondrial localization (Fig. 3C, bottom). In contrast to CYP24A1, CYP27B1 protein expression was not changed in response to either vehicle or 1α,25(OH)2D3 administration at physiological (MW) and supraphysiological (BW) doses (Fig. 3D). In addition, the CYP27B1 protein was expressed mainly in the mitochondrial compartment of the regenerative muscle fibers and extracellular matrix (Fig. 3E).

**Skeletal Muscle Regenerative Capacity in Responses to Intramuscular Administration of 1α,25(OH)2D3**

**Decreased regenerative muscle fiber CSA.** Hematoxylin and eosin-stained sections illustrated the presence of small regenerative muscle fibers in 1α,25(OH)2D3-administered groups at both physiological (MW) and supraphysiological (BW) doses compared with the control group (Fig. 4A). The number of regenerative muscle fiber size <600 μm² significantly increased in 1α,25(OH)2D3 administration at supraphysiological (BW) dose compared with vehicle group (49 ± 10 fibers vs. 251 ± 53 fibers, P < 0.01) (Fig. 4B). To support this notion, the CSA histogram revealed a leftward shift of regenerative muscle fiber CSA size <600 μm² in 1α,25(OH)2D3 administration at supraphysiological (BW) dose compared with vehicle group (Fig. 4C).
Decreased satellite cell differentiation and regenerative muscle fiber formation. Following the observation of increased number of regenerative muscle fibers size $<600 \, \mu m^2$, we investigated the expression level of proteins related to satellite cell differentiation (myogenin) that are required for developing nascent myotubes during skeletal muscle regeneration. Myogenin-positive nuclear localization in regenerative muscle fibers is illustrated in Fig. 5A (arrows). The qualitative expression of myogenin-positive nuclei in control, vehicle-, and $1\alpha,25(OH)_2D_3$-administered groups were demonstrated in Fig. 5B. The quantitative analysis from immunohistochemical staining revealed a significant decrease in myogenin-positive nuclei/field during skeletal muscle regeneration in the $1\alpha,25(OH)_2D_3$-administered group at supraphysiological (BW) dose compared with vehicle-administered group ($8 \pm 1$ vs. $14 \pm 2$ positive nuclei/field) ($P < 0.05$) (Fig. 5C). This result was confirmed by Western blot analysis with a suppressive of myogenin protein expression after $1\alpha,25(OH)_2D_3$ administration at supraphysiological (BW) dose (Fig. 5D). In addition, results from the in vitro study of C2C12 myotubes (Fig. 5E) supported in vivo results that myogenin protein expression was significantly decreased to $63.4 \pm 6.2\%$ after $1\alpha,25(OH)_2D_3$ treatment at $10 \, nM$ compared with the vehicle-treated group ($P < 0.01$) (Fig. 5F).

To further clarify that the suppression of satellite cell differentiation led to an impairment in skeletal muscle regeneration, we examined EbMHC protein expression that is required for de novo fiber formation. EbMHC protein localization and expression in regenerative muscle fibers is illustrated in Fig. 6A (arrows). Representative images were captured at $\times 400$ magnification; scale bars $= 20 \, \mu m$. C: CYP24A1 protein was expressed predominantly in the nucleus of C2C12 myoblasts and colocalized with VDR after $1\alpha,25(OH)_2D_3$ treatment at $10 \, nM$ for $24 \, h$. Barely overlapping with ATP synthase (complex V) subunit $\alpha$ (OxPhos) was detected in C2C12 myoblasts under the same experimental condition. Representative images were captured at $\times 200$ magnification; scale bars $= 100 \, \mu m$. D: Western blot analysis revealed CYP27B1 protein expression in control, vehicle-administered, and $1\alpha,25(OH)_2D_3$-administered groups ($n = 6$/group). GAPDH was used as a loading control. E: CYP27B1 protein is predominantly localized in the mitochondrial compartment of regenerative muscle fibers (OxPhos positive-stained) and the extracellular matrix. Representative images were captured at $\times 200$ magnification (cropped); scale bars $= 20 \, \mu m$. $1\alpha,25(OH)_2D_3$ (MW) and (BW) represent dose at $1\, \mu g/kg$ relative to TA muscle wet weight (physiological dose) and $1 \, \mu g/kg$ relative to mouse body weight (supraphysiological dose), respectively.
significantly suppressed after intramuscular administration of 1\(\alpha\),25(OH)\(_2\)D\(_3\) at supraphysiological (BW) dose compared with the vehicle-administered group as determined by quantitative expression analysis (3.9 ± 1.1 vs. 8.6 ± 1.7%) (P < 0.05) (Fig. 6C). These results were confirmed by Western blot analysis that demonstrate a suppressive effect on EbMHC protein expression after 1\(\alpha\),25(OH)\(_2\)D\(_3\) administration at supraphysiological (BW) dose (Fig. 6D). Furthermore, treatment of C2C12 myotubes with 1\(\alpha\),25(OH)\(_2\)D\(_3\) led to a dose-dependent suppression of EbMHC protein expression (Fig. 6, E and F). The quantitative analysis revealed significantly decreased EbMHC protein expression to 36.1 ± 4.4% after C2C12 myotubes were treated with 10 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\) compared with the vehicle-treated group (P < 0.001) (Fig. 6F). Taken together, these data suggest that intramuscular administration of 1\(\alpha\),25(OH)\(_2\)D\(_3\) at supraphysiological (BW) dose led to impaired muscle regenerative capacity via suppression of satellite cell differentiation and EbMHC protein expression.

No change in regenerative muscle protein synthesis and myostatin expression. To determine whether 1\(\alpha\),25(OH)\(_2\)D\(_3\) affects muscle protein synthesis during skeletal muscle regeneration, we examined its effect on the expression of p70 S6K1, 4E-BP1, and Akt that represent a major protein synthesis pathway in skeletal muscle. Western blot analysis demonstrated that phosphorylated protein/total protein levels of p70 S6K1 (Fig. 7A), 4E-BP1 (Fig. 7B), and Akt (Fig. 7C) were not significantly different in either administered condition [vehicle- or 1\(\alpha\),25(OH)\(_2\)D\(_3\)-administered groups]. To support this notion, there was also no difference in expression of mature myostatin protein compared between the vehicle- and 1\(\alpha\),25(OH)\(_2\)D\(_3\)-administered groups (Fig. 7D). These results suggest that an impairment of regenerative muscle capacity originated from the suppression of satellite cell differentiation and regenerative muscle fiber formation rather than decreased muscle protein synthesis.

No fiber-type transition. Recent studies in cell culture provided evidence that 1\(\alpha\),25(OH)\(_2\)D\(_3\) treatment induced increased type II muscle fibers (16). Here, we investigated whether intramuscular administration of 1\(\alpha\),25(OH)\(_2\)D\(_3\) could alter regenerative muscle fiber phenotype. The fiber-type distribution of mouse TA muscle between control and regenerative muscle are illustrated in Fig. 8A. The expression of fast fibers was higher than slow fibers in both normal and regenerative TA muscles. The results demonstrated no significant increase in fast fiber phenotype in any administered doses of 1\(\alpha\),25(OH)\(_2\)D\(_3\) in both myofibrillar (Fig. 8B) and cytosolic protein fractions (Fig. 8C). In contrast, there was a trend to increase slow MHC in regenerative muscle after administration of 1\(\alpha\),25(OH)\(_2\)D\(_3\) at supraphysiological (BW) dose in myofibrillar protein fraction (Fig. 8B). However, slow MHC protein expression was almost undetectable in cytosolic protein fraction (Fig. 8C).

Increased connective tissue formation. Extracellular matrix remodeling is an essential factor during skeletal muscle regeneration to provide the connective tissue support. Delayed muscle regeneration could lead to an increase in fibrous tissue formation. Connective tissue formation was significantly increased during skeletal muscle regeneration and further increased when 1\(\alpha\),25(OH)\(_2\)D\(_3\) at supraphysiological (BW) dose was intramuscularly administered as determined by vimentin.
protein expression (a marker of muscular fibrosis) (Fig. 9A). Quantitative analysis revealed an approximate fourfold increase in vimentin protein expression during skeletal muscle regeneration compared with control. Moreover, vimentin protein expression was significantly increased after 1α,25(OH)₂D₃ administration at supraphysiological (BW) dose compared with the vehicle group (18.7 ± 2.6% vs. 12.6 ± 1.0%) (P < 0.05). The expressions of vimentin protein in control and regenerative muscle with either vehicle or 1α,25(OH)₂D₃ administered at both physiological (MW) and supraphysiological (BW) doses was confirmed with Western blot analysis (Fig. 9C).

No change of capillary density. Revascularization is required during skeletal muscle regeneration to deliver nutrients and oxygen supply for functional recovery. Immunostaining of CD31, an endothelial cell marker that is used to determine angiogenesis is illustrated in Fig. 9D. In control muscle, the percentage of CD31 area expression was ~3.1 ± 0.2% and increased to 4.3 ± 0.2, 4.5 ± 0.2, and 4.5 ± 0.5% during skeletal muscle regeneration after administrations of vehicle and 1α,25(OH)₂D₃ at physiological (MW) and supraphysiological (BW) doses, respectively. However, there was no significant difference between vehicle- and 1α,25(OH)₂D₃-administered groups (Fig. 9E). CD31 protein expression in control, vehicle-, and 1α,25(OH)₂D₃-administered groups was confirmed with Western blot analysis (Fig. 9F).

DISCUSSION

In the present study, we examined the action of the bioactive form of vitamin D₃ [1α,25(OH)₂D₃] via intramuscular administration during skeletal muscle regeneration. The significant findings of our work are the following: 1) regenerative muscle expressed VDR and vitamin D₃ metabolizing enzyme.
(CYP24A1 and CYP27B1) proteins, which suggest the local metabolism of vitamin D3 in skeletal muscle during regenerative process; 2) an increased VDR protein expression in regenerative muscle after intramuscular administration of 1α,25(OH)2D3 was verified with VDR (D-6) antibody (this antibody was reported as highly sensitive antibody to detect VDR protein expression in skeletal muscle tissue); and 3) intramuscular administration of 1α,25(OH)2D3 at physiological dose (relative to TA MW) and supraphysiological dose (relative to mouse BW) into regenerative muscle had differential effects on skeletal muscle regenerative capacity and muscular fibrosis but not angiogenesis.

Expression of VDR protein is currently underdebated and contradictory results have been reported in normal skeletal muscle. Western blot analysis using VDR (D-6), which has the highest sensitivity to VDR, to verify VDR protein expression has been undetectable (30) and detectable (9) in whole muscle lysate. Moreover, VDR protein was expressed in normal skeletal muscle with immunohistochemistry using VDR (D-6) antibody (9), but this evidence has not been reported in the other investigation (31). Although, VDR protein was barely detectable in normal skeletal muscle, it was substantially expressed and localized in regenerative muscle fibers during skeletal muscle regeneration using VDR (H-81) antibody (26). In the present study, we further confirmed that VDR protein was expressed during skeletal muscle regeneration using highly sensitive VDR (D-6) antibody (29). Both VDR (D-6) and VDR (H-81) antibodies were raised against identical amino acids 344 – 424 of VDR of human origin, except the host species of VDR (D-6) was mouse monoclonal and VDR (H-81) was rabbit polyclonal. The specificity of VDR (H-81) was verified with VDR (D-6) antibody on its ability to detect VDR protein in regenerative muscle using immunohistochemical analysis. The results confirmed the expression of VDR protein in the central nuclei of regenerative muscle fibers can be detected with both VDR (H-81) and VDR (D-6) antibodies.
Skeletal muscle regeneration is the self-repairing process after muscle injury in which the regenerative capacity relies on the function of satellite cells (10). However, whether endogenous VDR protein is expressed in satellite cells is currently unknown. Here, we demonstrated that satellite cells expressed VDR protein during skeletal muscle regeneration as demonstrated by the colocalization of Pax7 and VDR proteins. This finding suggests the possible direct action of vitamin D3 on myogenesis during the regenerative process and further extends the knowledge on VDR protein expression in regenerative muscle (26). To support this notion, the presence of endogenous VDR protein expression was reported in the nucleus of C2C12 myoblasts (26) and primary myotubes isolated from skeletal muscle fibers (9), suggesting satellite cells could be the source of VDR after skeletal muscle injury.

The intramuscular administration of 1α,25(OH)2D3 was performed in this study to verify the direct action of 1α,25(OH)2D3 at the injured site. The beneficial effect of intramuscular administration of the bioactive form of vitamin D3 was the ability to control the concentration of 1α,25(OH)2D3 at the administered site, maximize the interaction of 1α,25(OH)2D3 to its receptor, and reduce the undesired systemic effect to the other tissues. In this study, physiological (MW) and supraphysiological (BW) doses of 1α,25(OH)2D3 with consecutive administration during skeletal muscle regeneration were investigated to clarify the differential effects of 1α,25(OH)2D3 dose. The results demonstrated that intramuscular administration of 1α,25(OH)2D3 at both physiological (MW) and supraphysiological (BW) doses significantly increased VDR protein expression in regenerative muscle. The effective dose which increased VDR protein expression was similar to the systemic dose (1 μg/kg BW) that is used to investigate calcemic function of 1α,25(OH)2D3 in the duodenum (a vitamin D3 sensitive tissue) (11). However, VDR protein expression was slightly increased (1.8-fold) in the supraphysiological (BW) dose administration compared with physiological (MW) dose. This result raised the possibility that regenerative muscle has a local control of vitamin D3 metabolism to regulate the response to high concentration of 1α,25(OH)2D3.

In vitro study in primary myotubes revealed that when the cells were treated with 1α,25(OH)2D3, CYP24A1 mRNA expression was significantly increased in a dose-dependent manner in response to 1α,25(OH)2D3 treatments (9). In the current investigation, CYP24A1 protein expression was increased after 1α,25(OH)2D3 was administered at supraphysiological (BW) dose, suggesting the local regulation of 1α,25(OH)2D3 concentration in vivo. The increase in CYP24A1 possibly decreased...
the toxicity of high concentration of 1α,25(OH)₂D₃ to regenerative muscle. Besides CYP24A1 protein expression, we investigated the expression level of CYP27B1 protein, a vitamin D₃ regulating enzyme, which was previously reported to be expressed in regenerative muscle (26). Administration of 1α,25(OH)₂D₃ at both physiological (MW) and supraphysiological (BW) doses did not change CYP27B1 protein level. This result could imply that the regulation of this enzyme was less affected by 1α,25(OH)₂D₃ concentration in vivo. In contrast, the regulation of CYP27B1 protein was dependent on the level of 25(OH)D₃ as demonstrated by luciferase reporter assays studied in both C2C12 myoblasts (8) and primary myotubes (9).

Impairments in muscle regenerative capacity were demonstrated after 1α,25(OH)₂D₃ administration at supraphysiological (BW) dose as CSA analysis revealed a significant increase in regenerating muscle fibers with a size <600 μm². A leftward shift of regenerative muscle fiber size <600 μm² could lead to a decrease in force production of regenerative muscle as the CSA is the major factor contributing to force development. This suppressive effect of 1α,25(OH)₂D₃ on skeletal muscle regeneration could involve either decrease satellite cell differentiation that led to impaired regenerative muscle fiber formation or suppression of protein synthesis signaling in regenerative muscle. Here, administration of 1α,25(OH)₂D₃ at supraphysiological (BW) dose induced a significant decrease in proteins related to satellite cell differentiation (myogenin) and regenerative muscle fiber formation (EbMHIC) during skeletal muscle regeneration. Suppression of these two proteins after 1α,25(OH)₂D₃ treatment in C2C12 myotubes in vitro supported the finding in vivo. These data suggest that satellite cell differentiation and regenerative muscle formation could be impaired after high concentration of 1α,25(OH)₂D₃ administration. However, the major pathway of skeletal muscle protein synthesis was not affected by 1α,25(OH)₂D₃ administration. Phosphorylation of Akt, P70 S6K1, and 4E-BP1 proteins relative to their total protein expressions were not altered. Moreover, myostatin protein expression during skeletal muscle regeneration was not influenced by 1α,25(OH)₂D₃ administration. Taken together, these results suggest that 1α,25(OH)₂D₃ could interfere with the process of muscle stem cell differentiation that leads to impaired regenerative muscle fiber formation rather than suppressing protein synthesis signaling in regenerative muscle.

Mouse TA muscle contains a majority of fast muscle fibers (>99%) (3); however, we observed a trend to increase slow MHC protein expression with no significant change of fast MHC when 1α,25(OH)₂D₃ was administered at supraphysiological (BW) dose compared with vehicle-administered dose. These data are in contrast to the previous reported that fast MHC when 1α,25(OH)₂D₃ was administered at supraphysiological (BW) dose at a 1 μg/kg relative to TA muscle wet weight (physiological dose) and 1 μg/kg relative to mouse body weight (supraphysiological dose), respectively.
tissue formation where excess connective tissue formation leads to muscular fibrosis. Related with an impairment of regenerative capacity after 1α,25(OH)2D3 administration at supraphysiological (BW) dose, vimentin protein expression was significantly increased. The presence of muscular fibrosis could be related to the action of 1α,25(OH)2D3 that acts as a suppressor of myogenin and EbMHC protein expression. Indeed, administration of activated 7-dehydrocholesterol via subcutaneous administration to rats increased prolyl-4-hydroxylase-β expression for 4 and 14 days following contusion, suggesting an excessive deposition of extracellular matrix protein after vitamin D3 treatment (27).

Angiogenesis is crucial for skeletal muscle regeneration to increase blood supply and nutrient delivery to regenerating muscle. Two proangiogenic growth factors (VEGF-A and FGF-1) were increased while myogenic and/or angiogenic inhibitors (FGF-2 and TIMP-3) were decreased in C2C12 myoblasts treated with 1α,25(OH)2D3 at MW and BW dose-administered groups. Western blot analysis revealed vimentin protein expression in control, vehicle-administered, and 1α,25(OH)2D3 at MW and BW dose-administered groups. The novelty of this work is the physiological dose. However, protein synthesis signaling, fiber-type composition, and angiogenesis in regenerative muscle were not affected by either a physiological or supraphysiological dose of 1α,25(OH)2D3. The novelty of this work is the finding of noncalcemic functions of 1α,25(OH)2D3 on regenerative capacity and muscular fibrosis during skeletal muscle regeneration. The results of the present study provide a significant contribution to the field regarding knowledge of the physiological functions of the bioactive form of vitamin D3 and vitamin D3 metabolizing enzymes on the regenerative process of skeletal muscle.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from the Olympus Bioimaging Center at the Faculty of Science, Mahidol University. We thank Professor Chumpol Pholphramnoo for kindly gifting the mouse C2C12 cell line and initial suggestions on developing this project. We also thank Kanokwan Subatcho and Narut Leerasantana for technical assistance. Proofreading of the manuscript by Assistant Professor Dr. Christopher Fry is appreciated.

GRANTS

This research project was supported by the Thailand Research Fund (TRF), Office of the Higher Education Commission, and Mahidol University (MRG5680061 to R. Srikuea) and the Faculty of Science, Mahidol University (to R. Srikuea).
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.S. conception and design of research; R.S. and M.H. performed experiments; R.S. and M.H. analyzed data; R.S. and M.H. interpreted results of experiments; R.S. prepared figures; R.S. drafted manuscript; R.S. and M.H. edited and revised manuscript; R.S. and M.H. approved final version of experiments; R.S. prepared figures; R.S. drafted manuscript; R.S. and M.H.

REFERENCES


