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Naohiko Hayakawa, Junko Fukumura, Hideyuki Yasuno, Kaori Fujimoto-Ouchi, and Hidemitsu Kitamura

ABSTRACT

Clinical trials involving in patients with osteoporosis have reported that activated vitamin D₃ (1α,25(OH)₂D₃, calcitriol) can prevent falling by acting on the skeletal muscles. However, pharmacological mechanisms of 1α,25(OH)₂D₃ with respect to skeletal muscle hypertrophy or atrophy are still poorly understood. Therefore, we examined changes in the expression of several related genes in human myotubes to test whether 1α,25(OH)₂D₃ influences hypertrophy and atrophy of skeletal muscle. Myotubes treated with 1α,25(OH)₂D₃ increased interleukin-6 (IL-6) expression and inhibited expression of tumor necrosis factor alpha (TNF-α), whereas the expression of insulin-like growth factor-1 (IGF-1) that is involved in muscle hypertrophy was not affected. However, 1α,25(OH)₂D₃ treatment significantly inhibited the expression of muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), ubiquitin ligases involved in muscle atrophy. The analysis of pathways using microarray data suggested that 1α,25(OH)₂D₃ upregulates AKT-1 by inhibiting the expression of protein phosphatase 2 (PP2A), a phosphatase acting on AKT-1, in the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, thereby inhibiting the expression of ubiquitin ligases. Thus, this study showed that 1α,25(OH)₂D₃ might have an inhibitory effect on the expression of MAFbx and MuRF1 in skeletal muscle and a suppressive effect on muscle degradation in patients with osteoporosis.

Skeletal muscle atrophy occurs when the normal balance between synthesis and degradation of muscle structural proteins is disturbed. Muscle atrophy F-box (MAFbx)/Atrogin-1 and muscle RING finger 1 (MuRF1) were identified as genes of two muscle-specific ubiquitin ligases (E3). These ligases are responsible for degradation of the muscle structural proteins in atrophied skeletal muscles that are caused by dietary restriction, aging, cancer, etc. These genes have been known to be significantly responsible for muscle atrophy because knockout of these genes reduces muscle atrophy caused by denervation (5, 19). It was also reported that atrophy of cultured myotubes can be suppressed by inhibiting the expression of MAFbx and MuRF1 using small interfering RNAs (10). Degradation of skeletal muscle is accelerated by increased MAFbx and MuRF1 expression induced by signals from inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (20). Systemic levels of these cytokines increase with age. IL-6 and TNF-α were considered to be markers of frailty and causative molecules of sarcopenia and cachexia in skeletal muscle (27).

Insulin-like growth factor-1 (IGF-1) strongly promotes myogenesis, while AKT-1 is activated by phosphorylation during the downstream process. When AKT-1 is activated, protein synthesis is increased by...
the S6 kinase system and the transcription factor forkhead box O (FOXO) is concurrently phosphorylated. Phosphorylated FOXO does not translocate to the nuclei, and consequently the expression of MAFbx and MuRF, both target genes of FOXO, is inhibited. According to one proposed model, in cases of muscle atrophy associated with disuse (e.g., induced by prolonged bed rest), decrease in IGF-1 causes inhibition of AKT-1 and dephosphorylation of FOXO. Dephosphorylated FOXO translocates into nuclei and promotes the expression of MAFbx and MuRF1, and subsequently accelerates degradation of muscle proteins (38).

Vitamin D₃ and its derivatives, which are used in clinical practices as a curative drug for osteoporosis, are considered to play a protective effect against bone fractures by preventing falls as well as having direct effects on bones. In addition, some clinical trials have demonstrated correlations between vitamin D supplementation and reduced risk of fractures (2–4, 16). Another report showed that administration of a vitamin D₃ derivative (alfacalcidol) improves muscle strength and body balance and significantly reduces the frequency of falls (14). These reports suggest that vitamin D₃ may have pharmacological effects on skeletal muscle. When administered to osteoporosis patients, it indirectly effects skeletal muscle and improves muscle strength or exercise capacity, and consequently suppresses falls and reduces fracture rates. However, the effects of 1α,25(OH)₂D₃ on skeletal muscle remain controversial and are poorly understood.

Therefore, in this study, we focused on muscle degradation and examined the effects of 1α,25(OH)₂D₃ on myotubes, differentiated from normal human skeletal myocytes.

MATERIALS AND METHODS

Reagents. 1α,25(OH)₂D₃ (for biochemistry) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). IL-6 was isolated and purified in our laboratory.

Cultivation of normal human myoblasts and differentiation to myotube. Human skeletal muscle cells (Pre-screened Human Adult Skeletal Muscle Cells), growth dedicated medium, and differentiation medium were purchased from Cell Applications, Inc. (San Diego, CA, USA). Myoblasts were cultivated according to the manufacturer’s protocol from growth to differentiation. Cells were propagated in T-75 flasks. They were seeded in collagen I-coated 12-well plates (Asahi Glass Co., Ltd., Tokyo, Japan) at 6.5 × 10⁴ cells/well in a growth-promoting medium. When the cells reached a subconfluent stage, the growth medium was switched with a differentiation medium and the cells were cultured for 6 days, when they differentiated to form myotubes and the agent was added. To confirm myotube formation by microscopic observation, the cultured cells were fixed in 100% of methanol and stained with methylene blue-basic fuchsin. 1α,25(OH)₂D₃ (final concentration of 10⁻⁸ mol/L), TNF-α (final concentrations of 0.1 ng/mL, 1 ng/mL, and 10 ng/mL), IL-6 (final concentrations of 1 ng/mL, 10 ng/mL, and 100 ng/mL), TNF-α + 1α,25(OH)₂D₃, and IL-6 + 1α,25(OH)₂D₃ were added to the myotubes and they were cultured for 24 to 72 h (n = 3).

Quantitative PCR. RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the SuperScript VILO™ MasterMix (Invitrogen, Carlsbad, CA, USA) according to each manufacturer’s protocol. Real-time PCR was performed according to the manufacturer’s protocol using the StepOne Plus System (Applied Biosystems, Foster City, CA, USA). Hs01041408_m1 (MAFbx), Hs00261590_m1 (MuRF1), Hs01547657_m1 (IGF-1), Hs01113624_g1 (TNF-α), and Hs00985639_m1 (IL-6) (Applied Biosystems) were used as TaqMan® probes and TaqMan® GAPDH Control Reagent (human) was used for the normalization of the control.

Microarray analysis. cDNA was synthesized using RNA (100 ng) sequences from the total RNA pool that was prepared from untreated myotubes and 1α,25(OH)₂D₃-stimulated myotubes (n = 3 for each group). The produced cDNA was converted to cRNA by in vitro transcription, and then was biotinylated. Synthesis was carried out using the 3’IVT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s recommended protocol. Biotin-labeled cRNA (12.5 μg) was added to the hybridization buffer and hybridized using the Human Genome U133 Plus 2.0 Array (Affymetrix) for 16 h. The arrays were washed, stained with phycoerythin using the GeneChip Fluidics Station 450, and scanned using the GeneChip Scanner 3000 7G. The acquired images were analyzed using the Affymetrix GeneChip Command Console software, and quantified using the Affymetrix Expression Console. The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and the transcription factor forkhead box O (FOXO) is concurrently phosphorylated. Phosphorylated FOXO does not translocate to the nuclei, and consequently the expression of MAFbx and MuRF, both target genes of FOXO, is inhibited. According to one proposed model, in cases of muscle atrophy associated with disuse (e.g., induced by prolonged bed rest), decrease in IGF-1 causes inhibition of AKT-1 and dephosphorylation of FOXO. Dephosphorylated FOXO translocates into nuclei and promotes the expression of MAFbx and MuRF1, and subsequently accelerates degradation of muscle proteins (38).

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Statistical analysis. All data are shown as means ± standard deviation (SD). Data were analyzed by unpaired t-tests, Dunnett’s multiple comparisons, or Tukey’s multiple comparisons using the SAS statistical analysis software package (SAS Institute Japan, Tokyo, Japan). A value of *P* < 0.05 was considered significant in all statistical analyses.

RESULTS

The effects of 1α,25(OH)2D3 on the expression of muscle atrophy and hypertrophy genes in human myotubes

At first, we induced human myotubes from skeletal muscle cells to investigate effects of 1α,25(OH)2D3 in vitro (Fig. 1). We measured the gene expression levels of MAFbx, MuRF1, and IGF-1 in human myotubes treated with 1α,25(OH)2D3 using quantitative real-time PCR. The expression levels of MAFbx and MuRF1, which are muscle-specific ubiquitin ligases, were significantly decreased 24 h after the addition of 1α,25(OH)2D3 (Fig. 2A). The expression level of IGF-1 was not affected (Fig. 2A). Decreased expression of MAFbx and MuRF1 was also observed at 72 h after addition of 1α,25(OH)2D3 (Fig. 2B).

The effects of 1α,25(OH)2D3 on induction of cytokine production in human myotubes

We measured the expression levels of IL-6 and TNF-α, which are inflammatory cytokines in human myotubes, after the addition of 1α,25(OH)2D3 using quantitative real-time PCR. The expression of IL-6 was significantly increased and the expression of TNF-α was considerably decreased at 24 h after the addition of 1α,25(OH)2D3 (Fig. 2C).

The effects of 1α,25(OH)2D3 on the expression of muscle atrophy and hypertrophy genes and induction of cytokine production in human myotubes stimulated by IL-6

In human myotubes stimulated by IL-6 for 24 h, the expression of MAFbx mRNA was significantly increased by the addition of 100 ng/mL of IL-6, and that of MuRF1 mRNA was significantly increased by the addition of 10 and 100 ng/mL of IL-6. The expression of IGF-1 was not affected by IL-6 (Fig. 3A), nor was the expression of TNF-α in skeletal muscle. The expression of IL-6 in skeletal muscle was significantly increased after the addition of 10 ng/mL and 100 ng/mL of IL-6 (Fig. 3B).

The expression of MAFbx was significantly decreased by the addition of a combination of 1α,25(OH)2D3 and 100 ng/mL of IL-6 at each time point compared with by the addition of IL-6 alone, and even with the control. The expression of MuRF1 was significantly decreased by the combination at 24 and 72 h compared with the addition of IL-6, and even with the control (Fig. 3C).

The expression of IGF-1 was not significantly affected by IL-6 for 24 h. The expression of TNF-α in skeletal muscle was significantly decreased by the addition of 1α,25(OH)2D3. The expression of IL-6 in skeletal muscle after the addition of IL-6 was not affected by the concurrent addition of 1α,25(OH)2D3 (Fig. 3D).

The effects of 1α,25(OH)2D3 on the expression of muscle atrophy and hypertrophy genes and cytokine production in human myotubes stimulated by TNF-α

In human myotubes stimulated by TNF-α at 24 h, the expression level of MAFbx mRNA was significantly increased after the addition of 0.1 ng/mL and 1 ng/mL of TNF-α and that of MuRF1 mRNA was significantly increased after the addition of TNF-α. The expression of IGF-1 was also significantly increased by the addition of 10 ng/mL of TNF-α (Fig. 4A). The expression of TNF-α was not increased by 0.1 and 1 ng/mL of TNF-α, but it was significantly increased
by 10 ng/mL of TNF-α. The expression of IL-6 in skeletal muscle was significantly increased by TNF-α (Fig. 4B).

The expression of MAFbx was significantly decreased by the combination of 0.1 ng/mL TNF-α and 1α,25(OH)₂D₃ compared with the addition of TNF-α alone. It was also significantly decreased compared with control at 24 and 72 h after the combined treatment. The expression of MuRF1 was significantly decreased by the combined treatment for all measurement times compared with the addition of TNF-α alone; as compared with the control, it was also significantly decreased at 72 h (Fig. 4C).

The expression of IGF-1 in skeletal muscle was not affected by the combined treatment with 0.1 ng/mL TNF-α and 1α,25(OH)₂D₃ for 24 h, and the expression of TNF-α was significantly decreased by the addition of 1α,25(OH)₂D₃. The expression of IL-6 in skeletal muscle was significantly increased by 0.1 ng/mL of TNF-α, and the increase was not affected by the concurrent addition of TNF-α and 1α,25(OH)₂D₃ (Fig. 4D).
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Analysis of 1α,25(OH)2D3 signaling pathway in human myotubes (microarray analysis)

We performed a microarray analysis comparing myotubes at 24 h after the addition of 1α,25(OH)2D3 and control myotubes, and performed a PI3K/AKT signaling pathway analysis (IPA) using the microarray data based on fold changes of 54675 genes. B-cell lymphoma 2 (BCL-2) was upregulated and protein phosphatase 2 (PP2A), JNK-interacting protein-1 (JIP1), β-CATENIN, and 14-3-3 protein (14-3-3) were downregulated after the addition of 1α,25(OH)2D3 (Fig. 5, Table 1).

DISCUSSION

Many recent studies have examined the effects of vitamin D3 on skeletal muscle with respect to muscle synthesis, proliferation, and differentiation, in which...
both positive and negative effects have been reported. Vitamin D₃ seems to act as a multifunctional regulator in skeletal muscle. During proliferation and differentiation, 1α,25(OH)₂D₃ upregulates AKT, Src and p38 mitogen-activated protein kinase (p38 MAPK), and thereby stimulates proliferation, survival, and differentiation of skeletal muscle cells (C2C12) (8). Suppression of differentiation and promotion of hypertrophy in myotubes have also been reported for vitamin D₃ (17). In myogenesis, 1α,25(OH)₂D₃ has been shown to promote protein synthesis through the AKT/mammalian target of rapamycin (mTOR) pathway in C2C12 myotubes (37). We studied the effects of 1α,25(OH)₂D₃ on muscle atrophy, for which there has been few report as vitamin D₃ effects on skeletal muscle, and focused specifically on MAFbx
1α,25(OH)2D3 downregulates MAFbx and MuRF1 in human myotubes. Our results indicate that MAFbx and MuRF1 mRNAs were inhibited in myotubes after the addition of 1α,25(OH)2D3, suggesting that vitamin D3 suppresses muscle atrophy.

A recent study of human subjects reported that when 1α,25(OH)2D3 is administered to hemodialysis patients, whole body muscle mass is significantly increased in male patients (28), and another study reported that when native vitamin D is administered to human adults, their IGF-1 levels in the blood increase (1). Our experimental results indicate that IGF-1, a muscle hypertrophy factor, was not directly affected by 1α,25(OH)2D3 in skeletal muscle. These results suggest that vitamin D3 may stimulate IGF-1 production in tissues other than skeletal muscle, and the induced IGF-1 might circulate in the blood and exert hypertrophic effects on muscle tissue or supportive effects on muscle function. A report that 1α,25(OH)2D3 enhanced the expression of IGF-1 in rat Schwann cells (35) also supports the possibility of indirect hypertrophic effects of vitamin D3 on skeletal muscle.

Skeletal muscle is an endocrine organ that produces cytokines and peptides called myokines (IL-6, IL-8, IL-15, brain-derived neurotrophic factor [BDNF], leukemia inhibitory factor [LIF]), which are reported to be profoundly involved in inflammatory processes via autocrine, paracrine, and endocrine pathways (32). Skeletal muscle is also an important source of cytokines that regulate skeletal muscle and immune function as well as systemic metabolism (7, 31).

**Table 1** Fold change values by PI3K/AKT signaling pathway analysis

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold Change (log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>1.615</td>
</tr>
<tr>
<td>PP2A</td>
<td>−1.083</td>
</tr>
<tr>
<td>JIP1</td>
<td>−1.386</td>
</tr>
<tr>
<td>β-CATENIN</td>
<td>−1.444</td>
</tr>
<tr>
<td>14-3-3</td>
<td>−2.123</td>
</tr>
</tbody>
</table>

Fig. 5 Graphical representation of the PI3K/AKT signaling pathway analysis using Ingenuity Pathway Analysis (IPA; accessed on August 22, 2014). The intergenic molecular relationships are shown at 24 h after the addition of 1α,25(OH)2D3. Red indicates upregulation (fold change > 1 (log2)) and green indicates downregulation (fold change < −1 (log2)).
Skeletal muscle expresses and secretes IL-6 and TNF-α during contraction. Substantial increases in IL-6 levels after exercise have been observed (42).

IL-6 in skeletal muscle acts on muscle stem cells and promotes muscle growth and myogenesis by regulating the proliferative capacity, but it also has been shown to promote muscle atrophy and muscle degradation (30). Moreover, IL-6 is reported to play a role in the regulation of differentiation of skeletal muscle via the signal transducer and activator of transcription 3 (STAT3) signaling pathway, and one study found that myotube formation is impaired in primary cultured myoblasts from IL-6 knockout mice (23). Skeletal muscle-derived IL-6 regulates proliferation and differentiation of muscle satellite cells and those of skeletal muscle, and functions as a regulator of skeletal muscle hypertrophy via muscle satellite cells. These IL-6 functions have been shown by knockout experiments using mice and examinations of C2C12 cells in which IL-6 is overexpressed (40). In our study, 1α,25(OH)2D3 increased the expression of IL-6 in skeletal muscle. This suggests that increased IL-6 is secreted from skeletal muscle and effects muscle satellite cells, resulting in hypertrophic effects. Another study indicated that skeletal muscle of rats induced IL-6 and TNF-α, when it was damaged by forced exercise, and the addition of native vitamin D3 inhibited the expression of IL-6 and TNF-α via regulation of MAPK and nuclear factor κB (NF-κB) (12). Increases in MAFbx and MuRF1 expression caused by IL-6 or TNF-α stimulation were also inhibited by 1α,25(OH)2D3 in our study. Accordingly, vitamin D3 displayed dual regulatory roles as both an inducer and inhibitor of cytokine or myokine expression in skeletal muscle.

In general, aging increases systemic TNF-α, which increases NF-κB, and TGF-β, which inhibits the AKT pathway; these processes result in muscle atrophy, which is characteristic of sarcopenia (36). TNF-α is likely to be involved in muscle wasting and weakness associated with inflammatory diseases (34), and TNF-α in signaling pathways involved in muscle degradation have been reported to increase the expression of MAFbx via p38 MAPK (25). TNF-α has also been reported to inactivate the PI3K/AKT pathway and induce degradation and apoptosis of L6 myotubes (41). In this study, the addition of 1α,25(OH)2D3 inhibited ubiquitin ligases and the expression of TNF-α in skeletal muscle simultaneously. In one model, TNF-α in the PI3K/AKT signaling pathway induces MAFbx by activating FOXO4 independently from AKT-FOXO1/3 signaling (29). Therefore, it is possible that 1α,25(OH)2D3 inhibits the expression of TNF-α and thereby inhibits FOXO activation, resulting in the inhibition of MAFbx and MuRF1 expression.

In skeletal muscle, PI3-kinase signaling regulates hypertrophy and atrophy. IGF-1 activates the PI3K/AKT signaling pathway and induces muscle hypertrophy (increased muscle mass, thickening and increase of muscle fibers). AKT is a protein kinase that is important in signaling pathways involved in protein synthesis and skeletal muscle growth. IGF-1 promotes phosphorylation of AKT and the activated AKT downregulates MAFbx and MuRF1 via downstream FOXO inhibition (39). On the other hand, when AKT is inactivated by dephosphorylation, the phosphorylation of downstream FOXO is inhibited, and FOXO translocates into cell nuclei to induce MAFbx and MuRF1, promoting muscle degradation (18). We performed a pathway analysis using microarray data to identify the role of 1α,25(OH)2D3 in the PI3K/AKT pathway that inhibits ubiquitin ligases. In this study, we confirmed that one gene was significantly up-regulated and 4 gene were down-regulated after the treatment of human myotubes with 1α,25(OH)2D3.

Our results showed that 1α,25(OH)2D3 inhibits protein phosphatase 2A (PP2A) in human myotubes. PP2A is known to inactivate AKT by dephosphorylation (44). This suggests a mechanism by which 1α,25(OH)2D3 activates AKT by downregulating PP2A and inhibiting the expression of ubiquitin ligases. This inhibition of PP2A may have a non-genomic effect because there are reports that 1α,25(OH)2D3 combined with vitamin D receptor (VDR) non-genomically activates MAPK, Src, and AKT (8, 9).

The addition of 1α,25(OH)2D3 has been shown in our microarray analysis to inhibit β-catenin that is involved in the Wnt signaling pathway. The Wnt signaling pathway has many intracellular signaling cascades and is reported to play an important role in myogenesis during pregnancy and after birth (13). Skeletal muscle does not grow normally in VDR knockout mice (15), suggesting that β-catenin inhibition caused by 1α,25(OH)2D3 may be involved in the regulation of myogenesis and differentiation via the VDR. In aging skeletal muscle, increased activity of Wnt signaling causes skeletal muscle fibrosis and muscular dystrophy, while inhibition of Wnt signaling suppresses fibrosis (6). Further studies are necessary to investigate the involvement of vitamin D3 in fibrosis and dystrophy of aging skeletal muscle.

This study also showed that 1α,25(OH)2D3 down-regulates JIP1, which is a known inhibitor of c-jun N-terminal kinase (JNK) (22). In an experimental
mouse, regulates metabolism by acting as a promoter or inhibitor depending on the conditions in vivo. Although it is not clear why 1α,25(OH)2D3 may influence the regulation of JNK signaling. One report shows that TNF-α induces the activation of JNK in myoblasts (43); therefore, it is possible that inhibition of the TNF-α expression caused by 1α,25(OH)2D3 observed in this study is indirectly involved in the regulation of JNK signaling.

14-3-3 proteins were downregulated by 1α,25(OH)2D3. 14-3-3 proteins regulate intracellular transport of glucose transporter type 4 (GLUT4) (33), and the possibility that 14-3-3 proteins regulate insulin response in skeletal muscle has been reported (24). Although the relationship between vitamin D3 and the regulator proteins of glucose metabolism in skeletal muscle is not known, 1α,25(OH)2D3 may influence the regulation of insulin signaling involving these proteins.

In the PI3K/AKT signaling pathway, 1α,25(OH)2D3 upregulated BCL-2, an anti-apoptotic molecule. BCL-2 has been reported to regulate autophagy induced by exercise in skeletal muscle (21), and to maintain skeletal muscle function in association with Naf-1, an autophagy regulator (11). These reports suggest that 1α,25(OH)2D3 is involved in the maintenance of skeletal muscle function including apoptosis via regulation of BCL-2.

In conclusion, this study demonstrated that 1α,25(OH)2D3 may have inhibitory effects on the expression of MAFbx and MuRF1, which encode ubiquitin ligases in skeletal muscle, and thereby suppresses muscle degradation. Furthermore, vitamin D3 is likely to be involved in the regulation of apoptosis, insulin responsiveness, myogenesis, and differentiation in functional regulation in skeletal muscle. Additional detailed studies, particularly those examining the relationship between vitamin D3 and nerves, will advance our understanding of vitamin D3-related mechanisms, especially those associated with the prevention of falls in osteoporosis patients treated with vitamin D3.

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