RESEARCH ARTICLE

1,25(OH)$_2$-vitamin D$_3$ enhances the stimulating effect of leucine and insulin on protein synthesis rate through Akt/PKB and mTOR mediated pathways in murine C2C12 skeletal myotubes

Jérôme Salles[1,2], Audrey Chanet[1,2], Christophe Giraudet[1,2], Véronique Patrac[1,2], Philippe Pierre[3,4,5], Marion Jourdan[6], Yvette C. Luiking[6], Sjors Verlaan[6], Carole Migné[1,2], Yves Boirie[1,2,7] and Stéphane Walrand[1,2]

[1] Clermont Université, Université d'Auvergne, Unité de Nutrition Humaine, Clermont-Ferrand, France
[2] INRA, UMR 1019, UNH, CRNH Auvergne, Clermont-Ferrand, France
[3] Centre d'Immunologie de Marseille-Luminy, Aix-Marseille Université, Marseille, France
[4] Institut National de la Santé et de la Recherche Médicale, U1104, Marseille, France
[5] Centre National de la Recherche Scientifique, UMR 7280 Marseille, France
[6] Nutricia Research, Utrecht, The Netherlands
[7] CHU Clermont-Ferrand, Service de Nutrition Clinique, Clermont-Ferrand, France

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Scope: In recent years, there has been a growing body of evidence pointing to an effect of vitamin D on muscle mass and function. Our aim was to investigate the combined effect of 1,25(OH)$_2$-vitamin D$_3$ (1,25(OH)$_2$D$_3$) with anabolic factors insulin and leucine on protein fractional synthesis rate (FSR) and regulation in the mouse C2C12 myotube.

Methods and results: After differentiation, myotubes were cultured in 1,25(OH)$_2$D$_3$ solutions at 0, 1, or 10 nM for 72 h. Cells were treated by L-[1–^{13}C]valine and puromycin in presence or not of leucine and insulin, and protein FSR was determined by measuring tracer enrichments and puromycin incorporation in proteins, respectively. Protein expression and phosphorylation state of insulin receptor (IR), Akt, GSK3, mTOR, p70 S6 kinase, rpS6, and 4EBP1 were measured by Western blot. Transcript levels of IR and 1,25(OH)$_2$D$_3$ receptor (VDR) were determined by qPCR. 1,25(OH)$_2$D$_3$ (10 nM) with leucine and insulin increased protein FSR in C2C12 myotubes (14–16%). IR and VDR mRNA expression was increased with 1,25(OH)$_2$D$_3$ treatment. The Akt/mTOR-dependent pathway was activated by insulin and leucine and further enhanced by 1,25(OH)$_2$D$_3$.

Conclusion: 1,25(OH)$_2$D$_3$ sensitizes the Akt/mTOR-dependant pathway to the stimulating effect of leucine and insulin, resulting in a further activation of protein synthesis in murine C2C12 skeletal myotubes.

Keywords:
Akt/mTOR pathway / C2C12 myotubes / 1,25(OH)$_2$-vitamin D$_3$ / Protein synthesis rate / Skeletal muscle

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Correspondence: Dr. Stéphane Walrand, Laboratoire de Nutrition Humaine, 58 rue Montalembert BP 321, 63009 Clermont-Ferrand Cedex 1, France
E-mail: swalrand@clermont.inra.fr
Fax: +33-4-73-60-82-55

Abbreviations: 1,25(OH)$_2$ D$_3$, 1,25(OH)$_2$-vitamin D$_3$; **4EBP1**, eukaryotic translation initiation factor-4E-binding protein 1; **AAs**, amino acids; **eEF2**, eukaryotic elongation factor 2; **FSR**, fractional synthesis rate; **GSK3**, glycogen synthase kinase 3; **IR**, insulin receptor; **p70S6K**, p70 S6 kinase; **rpS6**, S6 ribosomal protein; **VDR**, vitamin D receptor; **VDRE**, VDR responsive element

1 Introduction

Vitamin D deficiency is increasing in prevalence worldwide, with potential impacts on health. In recent years, there has been a growing body of evidence pointing to a negative effect of vitamin D deficiency on muscle mass, morphology, and function, especially in the elderly. Several observational studies on elderly populations have reported a positive association between 25(OH) vitamin D plasma concentrations and physical performance [1–3] and an inverse association with risk of falling [4]. In addition, supplementation with vitamin D resulted in beneficial effects on muscle strength and risk of falling in older adults with low 25(OH) vitamin D status [5–7]. Moreover, at tissue level, vitamin D deficiency has been associated with proximal myopathy and muscle fibre atrophy, which can be reversed by the administration of vitamin D [5, 8].

In tissues, 1,25$(OH)_2$ vitamin D_3 (1,25$(OH)_2D_3$), the biologically active form of vitamin D, acts through genomic and nongenomic pathways. The fast nontranscriptional response to vitamin D involves stimulation of transmembrane second-messenger systems while the expression of target genes is under the control of its specific nuclear receptor (vitamin D receptor, VDR). The presence of the VDR in skeletal muscle cells is currently under debate [9] mainly due to the quality of several antibodies directed against the VDR protein [10]. Nevertheless, the identification of the VDR in myoblasts, myotubes, and skeletal muscle using different methods (transcript cloning and sequencing, immunoblotting, immunocytochemistry) [11–13] together with the recent finding of an active form of CYP27B1, the enzyme converting circulating 25(OH) vitamin D_3 into 1,25$(OH)_2D_3$, in C2C12 cells [12] argues for a direct effect of 1,25$(OH)_2D_3$ on muscle tissue. Further support comes from previous reports showing that 1,25$(OH)_2D_3$ could affect cell proliferation and differentiation in both primary chick and C2C12 myoblasts [11, 12, 14, 15] and could improve palmitate-induced insulin resistance in C2C12 myotubes [16]. However, despite the described association between muscle mass and circulating 25$(OH)D_3$, the effect of vitamin D on myotube cell growth and metabolism have never yet been explored.

Myotube cell growth and maintenance is largely dependent on protein synthesis stimulation involving the translation initiation and elongation steps. Translation initiation is controlled by the Akt/mTOR pathway leading to the phosphorylation of several downstream targets such as GSK3 (glycogen synthase kinase 3), p70 S6 kinase (p70S6K) and 4EBP1 (eukaryotic translation initiation factor-4E-binding protein-1). The elongation process involves elongation factors including eEF2 (eukaryotic elongation factor 2) whose phosphorylation inhibits its binding to the ribosome and therefore the elongation step.

Anabolic factors such as insulin and leucine are important activators of the Akt/mTOR pathway. Insulin binding to its receptor classically promotes mTOR signaling and muscle protein anabolism [17]. In addition, leucine acts as a signal nutrient in promoting protein synthesis in skeletal muscle and adipose tissue via mTOR pathway activation, opening up potential perspectives in age-related sarcopenia [18]. Muscle protein loss during aging may be partly explained by a decreased ability of muscle to respond appropriately to anabolic stimuli provided by dietary proteins [19, 20] through a decreased postprandial response of protein synthesis to physiological concentrations of amino acids (AAs), especially leucine [21, 22], and insulin [23]. Interestingly, vitamin D deficiency has been frequently associated with insulin resistance in vivo [24], while vitamin D treatment has been associated with increased expression of the insulin receptor (IR) in various tissues including skeletal muscle [25, 26]. The literature also includes intriguing reports of common effects of vitamin D deficiency and aging per se on muscle. Taken together, these data suggest that vitamin D could interfere with anabolic mediators, i.e. leucine and insulin, possibly explaining the negative association of vitamin D deficiency with muscle mass and function described in elderly people. To test this hypothesis, we investigated the combined effect of 1,25$(OH)_2D_3$ with anabolic factors insulin and leucine on protein fractional synthesis rate (FSR) and its regulation pathways in the mouse C2C12 myotube cell line as an in vitro model for skeletal muscle.

2 Materials and methods

2.1 Chemicals

1,25$(OH)_2$ Vitamin D3 (calcitriol) was purchased from Cayman Europe (Tallinn, Estonia). Insulin, leucine, DMEM containing 4.5 g/L glucose, antitotal p38 antibody, protease-inhibitor cocktail, puromycin, and primers were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Leucine-free DMEM was prepared by BioWest (Nuaillé, France). Fetal bovine and heat-inactivated horse sera, trypsin-EDTA, PBS, penicillin/streptomycin were obtained from PAA (Pasching, Austria). L-[1–^{13}C]valine was purchased from Euriso-top (Saint-Aubin, France). Primary antibodies were obtained from the following sources: antitotal IR β, antiphospho Akt (Ser473), antitotal Akt, antiphospho GSK-3β (Ser9), antitotal GSK-3β, antiphospho mTOR (Ser2448), antitotal mTOR, antiphospho p70 S6 kinase (Thr389), antitotal p70 S6 kinase, antiphospho S6 ribosomal protein (Ser235/236), antitotal S6 ribosomal protein, antiphospho 4EBP1 (Ser65), antitotal 4EBP1, antiphospho eEF2 (Thr56), and antitotal eEF2 (Cell Signaling Technology, Ozyme distributor, Saint-Quentin-en-Yvelines, France); antiphospho IR β (Tyr1158) (Abcam, Paris, France). Rabbit antivitamin D_3 receptor was purchased from Epitomics (Euromedex distributor, Mundolsheim, France) and was directed against the N-terminal region of the human VDR (amino acid positions 8 to 48). Mouse antipuromycin mAb (clone 12D10) is described

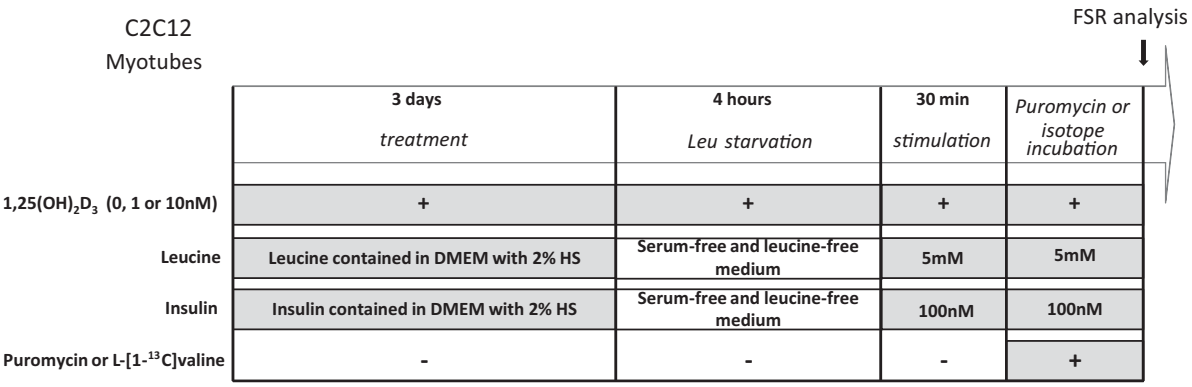

Figure 1. Schematic diagram of the experimental design. After 5 days of differentiation, myotubes were treated with 0, 1, or 10 nM of $1,25(OH)_2D_3$ for 72 h. Cells were serum and leucine-starved for 4 h and then stimulated with a mix of insulin + leucine (100 mM and 5 mM final concentrations, respectively) for 30 min. Cells were therefore studied in stimulated (insulin + leucine) or unstimulated (without insulin and leucine) conditions and treated with 0, 1, or 10 nM of $1,25(OH)_2D_3$.

in [27, 28]. Swine antirabbit and goat antimouse horseradish peroxidase conjugated secondary antibodies were purchased from DAKO (Trappes, France). SuperScript® III reverse transcriptase, random hexamer and oligo dT primers were from Invitrogen (Life Technologies, Saint-Aubin, France). Tri-Reagent was purchased from Euromedex (Mundolsheim, France). Rotor-Gene SYBR Green PCR master mix was purchased from Qiagen (Courtaboeuf, France).

2.2 Muscle cell culture

C2C12 myoblasts (ATCC, Manassas, VA) were grown to 80–90% confluence in DMEM supplemented with 10% foetal calf serum at 37°C in a 5% CO_2-humidified atmosphere. They were then induced to differentiate into myotubes by switching to DMEM containing 2% heat-inactivated horse serum (Differentiation Medium, DM). After 5 days of differentiation, early and late markers of myogenic differentiation were expressed (Supporting Information Fig. 1). At this differentiation stage, myotubes were cultured for 3 days with DM containing either 0, 1, or 10 nM $1,25(OH)_2D_3$ dissolved in ethanol. The final concentration of ethanol in the media was 0.1% as previously described by Garcia et al. and Srikuea et al. [11, 12]. All the following experiments were performed on day 8.

2.3 Stable isotope measurement of protein synthesis rates

The study design and conditions tested are described in Fig. 1. DM containing $1,25(OH)_2D_3$ was replaced by the same medium depleted of leucine and serum. After 4 h of leucine and serum starvation, a mix of insulin (100 nM final concentration) + leucine (5 mM final concentration) was added to the C2C12 myotubes. L-[1–^{13}C]valine was added 30 min later, and incubation was continued for 50 min. Control myotubes were treated with appropriate vehicles. Cells were then rinsed twice with PBS and treated with the lysis buffer described in the Western blot section. Cell proteins were hydrolysed using 6 M HCl (110°C for 24 h), and the AAs in the hydrolysate were purified by cation exchange chromatography (Dowex 50W 8X; Bio-Rad, Hercules, CA). AAs were derivatized as their N-acetyl-propyl, and isotopic enrichment was measured by gas chromatography-combustion-isotope ratio mass spectrometry (μGas System, Fisons Instruments, VG Isotech, Middlewich, United Kingdom). L-[1–^{13}C]valine enrichments in tissue fluid were assessed on a GC-mass spectrometer (Hewlett-Packard 5971A; Hewlett-Packard Co., Palo Alto, CA) and used as precursor pool enrichment for the FSR calculations. FSR was calculated as previously described [29, 30]. Protein FSRs were calculated using the following equation:

$$FSR = (Ei \times 100)/(Ep \times t) \quad (1)$$

where Ei represents enrichment as the atom percentage excess of L-[1–^{13}C]valine derived from valine from proteins at time t (minus basal enrichment); Ep is the mean enrichment in the precursor pool (cellular fluid L-[1–^{13}C]valine); t is the incorporation time in hours. Data (%/h) are expressed as a percentage of the data obtained in cells not treated with $1,25(OH)_2D_3$ and not stimulated by insulin + leucine.

2.4 Western blot analysis and measurement of protein synthesis rates with puromycin

After 30 min of insulin + leucine treatment, 1 μM of puromycin was added to the culture medium, as previously described [28]. C2C12 myotubes were incubated for 30 min and then homogenized in an ice-cold lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM NaPPi, 25 mM β-glycerophosphate, 100 mM NaF, 2 mM Na orthovanadate, 10% glycerol, 1% Triton X-100, Sigma-Aldrich)

Table 1. Primer sequences for the quantitative analysis of gene expression

Gene name	NCBI accession number	Forward and reverse primers
VDR (Vitamin D Receptor)	NM_009504.4	For 5′-CACCTGGCTGATCTTGTCAGT-3′ Rev 5′-CTGGTCATCAGAGGTGAGGTC-3′
IR (Insulin Receptor)	NM_010568.2	For 5′-TTTGTCATGGATGGAGGCTA-3′ Rev 5′-CCTCATCTTGGGGTTGAACT-3′
18S rRNA	NR_003278.1	For 5′-CGGCTACCACATCCAAGGAA-3′ Rev 5′-GCTGGAATTACCGCGGCT-3′

containing a protease-inhibitor cocktail (1%). Puromycin incorporation into nascent polypeptides was measured by separating 15 μg of denaturated proteins by SDS-PAGE on a polyacrylamide gel until the dye front was 1.5 cm from the bottom of the gel [27]. Values (arbitrary units) are expressed as a percentage of the values obtained in cells not treated with $1,25(OH)_2D_3$ and not stimulated by insulin + leucine. Protein phosphorylation level and content were measured by loading 50 μg of denaturated proteins onto a gel. SDS-PAGE separated proteins were transferred to a polyvinylidene membrane (Millipore, Molsheim, France). Immunoblots were blocked with TBS-Tween-20 0.1% containing 5% dry milk and then probed overnight at 4°C with primary antibodies. After several washes with TBS-Tween-20 0.1%, immunoblots were incubated with a horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. The immune reactive strips or whole lanes were visualized by chemiluminescence (ECL Western Blotting Substrate, Pierce, IL). Luminescent secondary antibodies were visualized using Biomax light film (Kodak, NY). The intensity of the strips or whole lanes was quantified by densitometry using Scion Image software (Scion Corporation, TX). The activation states were evaluated based on the ratio of phosphorylated protein-to-total protein expression. Expression of the total amount of p38 was used to normalize protein loading between samples [31].

2.5 Quantitative RT-PCR analysis

Total RNA was extracted using Tri-Reagent according to the manufacturer's instructions. RNA was quantified by measuring optical density at 260 nm. The concentrations of mRNAs corresponding to genes of interest were measured by reverse transcription followed by RT-PCR using a Rotor-Gene Q system (Qiagen, France). Five μg of total RNA was reverse-transcribed using SuperScript III reverse transcriptase and a combination of random hexamer and oligo dT primers. cDNAs were diluted 1:60 before PCR analysis. PCR amplification was performed in a 20 μL total reaction volume. The RT-PCR mixture contained 5 μL diluted cDNA template, 10 μL 2× Rotor-Gene SYBR Green PCR master mix, and 0.5 μM forward and reverse primers. The amplification profile was initiated by a 5-min incubation at 95°C to activate HotStarTaq Plus DNA Polymerase, followed by 40 cycles of two steps: 95°C for 5 s (denaturation step) and 60°C for 10 s (annealing/extension step). Relative mRNA concentrations were analyzed using Rotor-gene software. Seven-fold serial dilutions from a mix of all undiluted cDNA were used for each target gene to construct linear standard curves from which the concentrations of the test sample were calculated. Table 1 lists the primers used for real-time PCR amplification. The expression level of 18S rRNA was used to normalize mRNA levels.

2.6 Statistical analysis

All data are presented as means ± SEM. A one-way analysis of variance was performed to test the effect of the experimental conditions. When a significant effect was detected, an a posteriori Fisher test was applied to locate pairwise differences between conditions. Statistical analysis was performed using StatView (version 4.02; Abacus Concepts, Berkeley, CA). Values of $p < 0.05$ were considered as significant.

3 Results

3.1 Treatment with $1,25(OH)_2D_3$ potentiates the effect of anabolic factors leucine and insulin on muscle protein synthesis

No morphological differences between C2C12 myotubes treated with $1,25(OH)_2D_3$ for 72 h and not treated C2C12 myotubes was noticed (Supporting Information Fig. 2). To examine the effect of $1,25(OH)_2D_3$ on protein synthesis, differentiated C2C12 myotubes treated with 0, 1, or 10 nM of $1,25(OH)_2D_3$ for 72 h were incubated with 100 nM of insulin and 5 mM of leucine for 30 min (stimulated condition). We first assessed protein synthesis by measuring stable isotope incorporation into free AA and proteins after adding a flooding dose of L-[1-^{13}C]valine to the culture media (Fig. 2A). As expected [32, 33], stimulation of C2C12 myotubes with anabolic factors, i.e. insulin + leucine, significantly increased protein synthesis rate by 14% compared to unstimulated cells. When stimulated myotubes were pretreated with different $1,25(OH)_2D_3$ concentrations for 72 h, protein synthesis was dose-dependently enhanced but was significantly different from control only at a dose of 10 nM $1,25(OH)_2D_3$ (+14% versus stimulated C2C12 myotubes not treated with $1,25(OH)_2D_3$, $p < 0.05$) (Fig. 2A). This

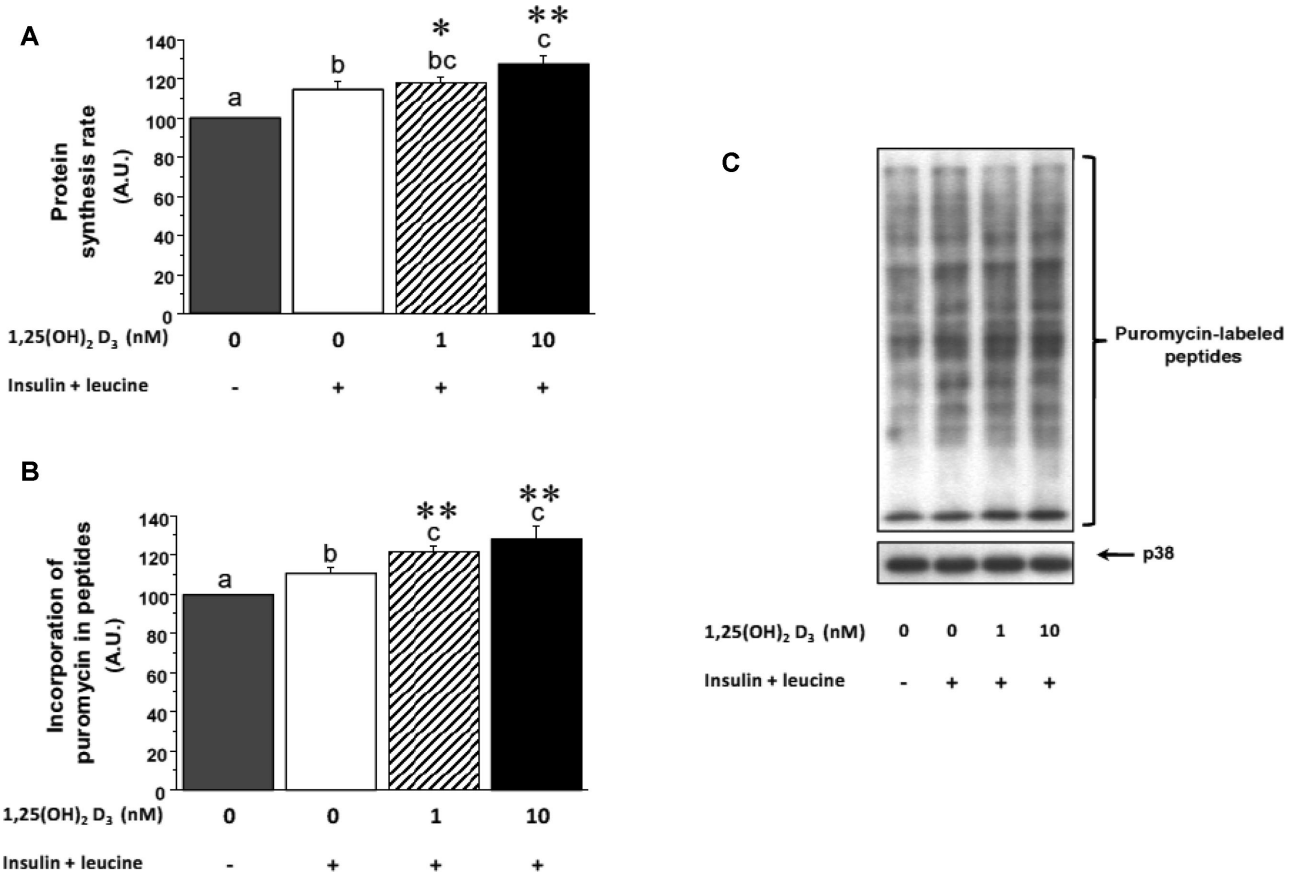

Figure 2. Effect of $1,25(OH)_2D_3$ on protein synthesis in C2C12 myotubes. Cells were incubated as described in the legend to Fig. 1. Protein synthesis was measured in myotubes after (A) a 50-min incubation with L-[1-^{13}C]valine, by measuring tracer enrichment, or (B) a 30-min incubation with puromycin, by quantifying incorporation of puromycine into peptides. (C) Representative immunoblots showing levels of puromycin-labeled peptides in different conditions. Expression of the total amount of p38 was used to normalize protein loading between samples. Values are expressed as a percentage of the values obtained in cells not treated with $1,25(OH)_2D_3$ and not stimulated by insulin + leucine. Data are expressed as means ± SEM ($n = 5$). Means sharing the same superscript letter are not significantly different (a ≠ b ≠ c for $p < 0.05$). *, statistically different from C2C12 myotubes not treated with $1,25(OH)_2D_3$ and not stimulated with insulin and leucine at $p < 0.01$. **, statistically different from C2C12 myotubes not treated with $1,25(OH)_2D_3$ and not stimulated with insulin and leucine at $p < 0.001$. A.U.: Arbitrary Unit.

observation suggests that $1,25(OH)_2D_3$ treatment is able to potentiate the stimulation of protein synthesis by anabolic factors in myotubes. To confirm these data, we prepared cell protein extracts in order to measure protein synthesis using an immunodetection method, i.e. puromycin incorporation in neosynthesized proteins. This new method also made it possible to determine protein synthesis rate and activation of key signaling pathways involved in this process using the same experimental samples. Cell stimulation with insulin and leucine significantly increased protein synthesis measured by the puromycin method compared to unstimulated myotubes (+ 11%, $p < 0.05$). C2C12 myotubes exposed to 1 and 10 nM $1,25(OH)_2D_3$ for 72 h exhibited a greater increase in protein synthesis in response to anabolic factors than untreated cells (+10% and +16% versus stimulated C2C12 myotubes not treated with $1,25(OH)_2D_3$, respectively, $p < 0.05$ Fig. 2B and C). Hence, comparable results were obtained for stable isotope incorporation into proteins and for puromycin incorporation into nascent polypeptides. Taken together, these data indicated that treatment with $1,25(OH)_2D_3$ enhanced the stimulatory effect of insulin and leucine on protein synthesis in C2C12 myotubes (Fig. 2A–C).

3.2 Treatment with $1,25(OH)_2D_3$ increases the stimulation of IR and Akt/mTOR pathway by anabolic factors in skeletal muscle cells

To further investigate the mechanism leading to the observed enhanced stimulatory effect of leucine and insulin on protein synthesis rate following treatment with 10 nM $1,25(OH)_2D_3$, we analyzed the activation state of intracellular signaling pathways controlling protein translation initiation and elongation. As previously described [32–34], adding

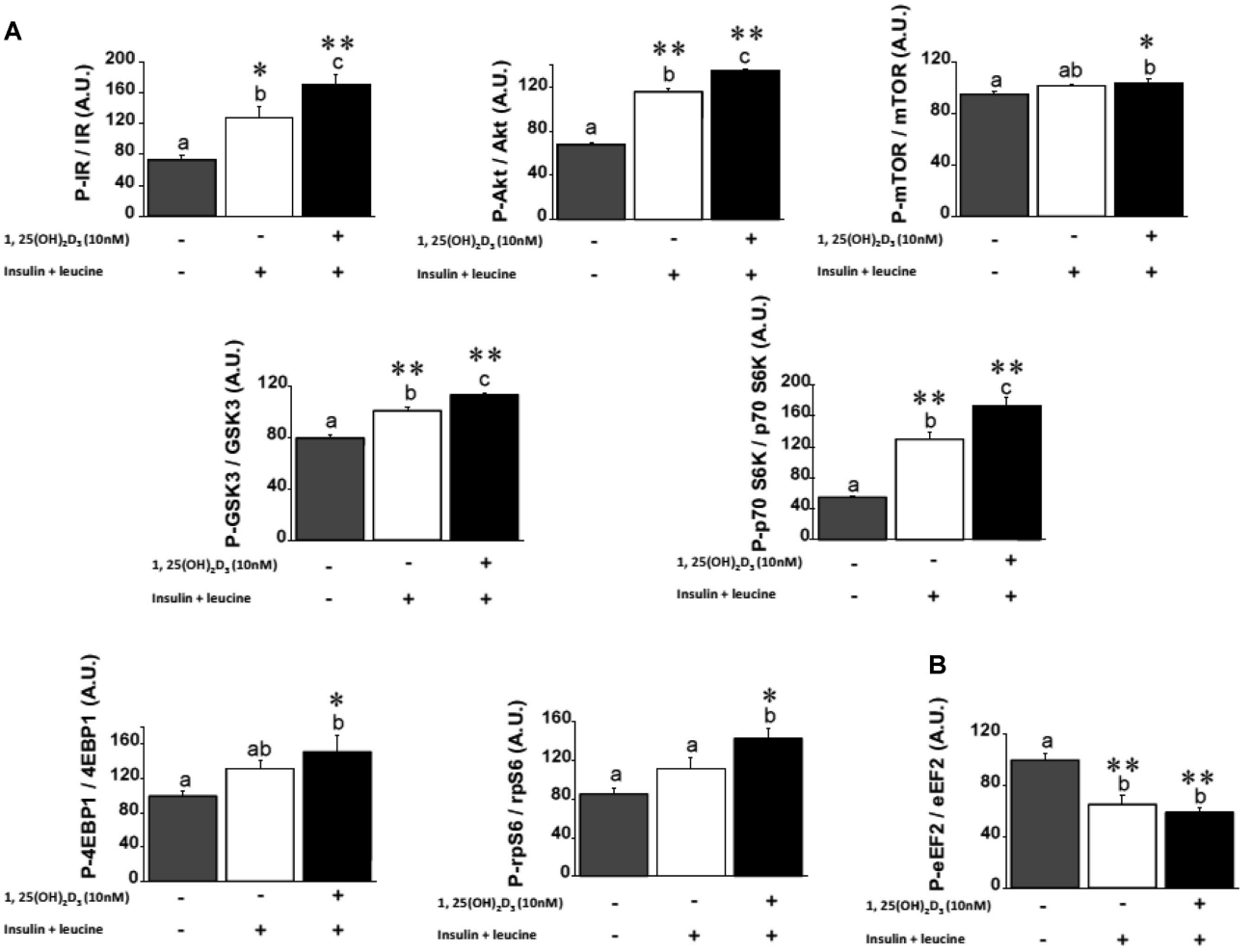

Figure 3. Effect of $1,25(OH)_2D_3$ on signal transduction pathways involved in the translation initiation (A) and elongation (B) steps in C2C12 myotubes. Cells were incubated as described in the legend to Fig. 1. Homogenates resulting from measurement of protein synthesis with puromycin labeling were used to assess phosphorylation state of (A) IR, Akt, mTOR, GSK3, p70 S6K, 4EBP1, rpS6 and (B) eEF2. A representative immunoblot illustrating the level of phosphorylated and total forms is shown Supporting Information Fig. 3. Data are expressed as means ± SEM. ($n = 5$). Means sharing the same superscript letter are not significantly different (a ≠ b ≠ c for $p < 0.05$). *, statistically different from C2C12 myotubes not treated with $1,25(OH)_2D_3$ and not stimulated with insulin and leucine at $p < 0.01$. **, statistically different from C2C12 myotubes not treated with $1,25(OH)_2D_3$ and not stimulated with insulin and leucine at $p < 0.001$. A.U.: Arbitrary Unit.

anabolic factors to the culture medium resulted in a significant activation of both the IR and the Akt/PKB controller compared to unstimulated controls (Fig. 3A). Additionally, our results showed that a 72-h exposure to $1,25(OH)_2D_3$ led to an increased ratio of phosphorylated-to-total IR (+33%; $p < 0.01$; Fig. 3A) compared to untreated stimulated controls, i.e. without $1,25(OH)_2D_3$. These data indicate a greater level of activation of the IR following combined incubation with $1,25(OH)_2D_3$ and anabolic factors compared to anabolic factors alone. This latter effect was accompanied by subsequent changes in the activation state of IR downstream effectors involved in protein translation initiation. Indeed, following $1,25(OH)_2D_3$ treatment, anabolic factor-induced protein phosphorylation levels were significantly higher for Akt (+16.5%), GSK3 (+12.5%), p70S6K (+32%), and rp S6 (+17%; Fig. 3A). There was no significant increase in the level of phosphorylated mTOR and 4E-BP1 after stimulation with insulin + leucine in C2C12 control cells (Fig. 3A). However, myotubes treated with $1,25(OH)_2D_3$ and stimulated with insulin + leucine showed a greater level of phosphorylated mTOR and 4E-BP1 than unstimulated C2C12 control cells (+9% and +52%, $p < 0.05$, respectively). As expected, our results indicated a significant reduction in the ratio of phosphorylated eEF2 relative to total eEF2 after adding insulin and leucine to the medium (−37%; Fig. 3B, [35]). However, treatment with $1,25(OH)_2D_3$ before stimulation with anabolic factors had no further effect (Fig. 2B).

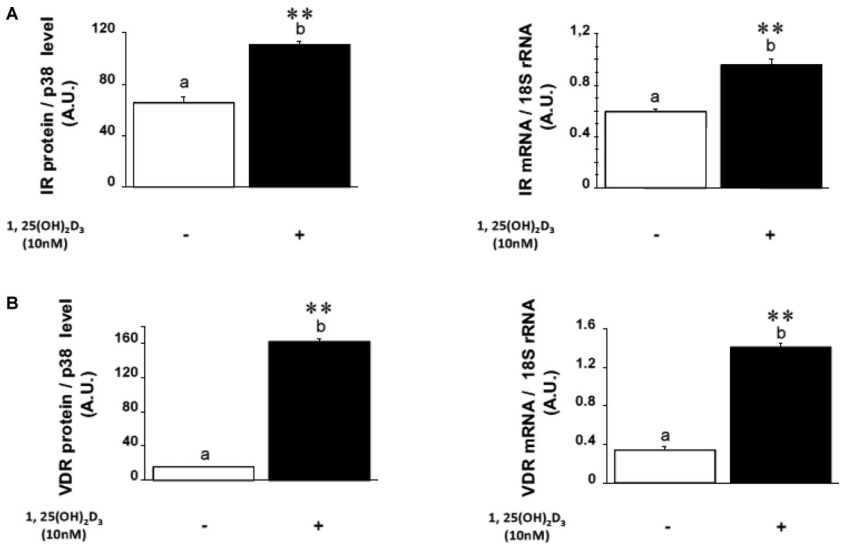

Figure 4. Effect of $1,25(OH)_2D_3$ on the expression of IR protein and mRNA (A) and VDR protein and mRNA (B). For protein-level analysis, cells were incubated as described in the legend to Fig. 1. Homogenates resulting from measurement of protein synthesis with puromycin labeling used to assess the protein content of IR and VDR by Western blotting. Expression of the total amount of p38 was used to normalize protein loading between samples. A representative immunoblot is shown Supporting Information Fig. 4. Data are expressed as means ± SEM. ($n = 5$). For mRNA-level analysis, 5-day-differentiated C2C12 myotubes were incubated for 72 h with or without 10 nM of $1,25(OH)_2D_3$. Total RNA was extracted, and IR and VDR mRNA levels were analyzed by quantitative RT-PCR and normalized by 18S rRNA. Data are expressed as means ± SEM ($n = 6$). Means sharing the same superscript letter are not significantly different (a ≠ b for $p < 0.05$). **, statistically different from C2C12 myotubes not treated with $1,25(OH)_2D_3$ at $p < 0.001$. A.U.: Arbitrary Unit.

3.3 $1,25(OH)_2D_3$ treatment stimulates IR and VDR expressions in skeletal muscle cells

To clarify how a 72-h $1,25(OH)_2D_3$ treatment is able to increase the level of activation/phosphorylation of the Akt/mTOR pathway, we measured the effect of $1,25(OH)_2D_3$ alone on gene and protein expression of IR, which is the first intermediate of this pathway. After 72-h exposure to 10 nM $1,25(OH)_2D_3$, C2C12 myotubes showed a significant increase in IR protein expression (+76%; Fig. 4A). This latter change could result from a significant increase in IR gene expression following $1,25(OH)_2D_3$ treatment, as observed here (+62%; Fig. 4A). In addition, treatment with $1,25(OH)_2D_3$ strongly increased the protein and gene expression of its own receptor, namely the VDR (+931% and +307%, respectively; Fig. 4B) compared to untreated controls. Taken together, these data show that $1,25(OH)_2D_3$ increases the transcriptional regulation of VDR and IR expression, which could enhance C2C12 myotube sensitivity to vitamin D and insulin, respectively.

4 Discussion

Despite the fact that muscle wasting and weakness are prominent features of nutritional rickets [36], a direct action of $1,25(OH)_2D_3$ on muscle metabolism has never yet been studied. At tissue level, $1,25(OH)_2D_3$ deficiency is associated with proximal myopathy and histological evidence of muscle fiber atrophy and protein loss that rapidly improve after the administration of $1,25(OH)_2D_3$ or its metabolites [8]. Protein turnover in any tissue is determined by the rates of protein synthesis and degradation, so alterations in either or both processes could be responsible for the effects of $1,25(OH)_2D_3$ in muscle. Only one previous report (carrying limitations), to use muscle preparations from 25-hydroxycholecalciferol [$25(OH)D_3$]-supplemented rats, suggests that this precursor performs a specific function within skeletal muscle to improve net muscle protein anabolism [37]. However, whether $1,25(OH)_2D_3$ or its metabolites act directly or indirectly through its numerous systemic properties on muscle tissue remains an open question. Therefore, this study was designed to specifically evaluate the mechanisms of $1,25(OH)_2D_3$ action on muscle cell anabolism. As muscle protein synthesis rate is positively regulated by several postprandial factors such as dietary leucine and insulin, we tested the effect of 1 and 10 nM $1,25(OH)_2D_3$ in a leucine + insulin-stimulated condition. Data obtained demonstrated that $1,25(OH)_2D_3$ at a concentration of 10 nM sensitizes the Akt/mTOR-dependent pathway to the stimulating effect of leucine + insulin, resulting in a further significant activation of protein synthesis in murine C2C12 myotubes. Therefore, $1,25(OH)_2D_3$ potentiates the effect of leucine + insulin on skeletal muscle anabolism.

This study found that adding $1,25(OH)_2D_3$ to C2C12 skeletal muscle cells increased the expression, i.e. transcript and protein levels, of the VDR. This observation was expected to some extent, since studies in humans and rats report

that $1,25(OH)_2D_3$ autoregulates the expression of the VDR gene through intronic and upstream enhancers [11, 38]. The VDR is an absolute determinant of the biological activity of $1,25(OH)_2D_3$ [39]. Thus, cell expression of the receptor is a requirement for the response, and the receptor concentration itself a key component of sensitivity to the vitamin as well. In the majority of cases where $1,25(OH)_2D_3$ acts as a positive gene regulator through its binding to the VDR, the liganded VDR heterodimerizes and forms a complex with other receptors like RXR that binds to specific DNA sequences, i.e. VDR-responsive element (VDR elements, VDRE), in the promoter area of target genes to control their expression. Interestingly, a computer search in the rat IR promoter revealed the existence of two candidate VDRE sequences located at −256/−219 bp and −653/−620 bp [25]. The candidate VDREs identified may respond to $1,25(OH)_2D_3$ via activation of the VDR, although this remains to be investigated. In addition to the VDR, other receptors are upregulated by $1,25(OH)_2D_3$ in muscle cells, such as the IR [26, 40]. Here, not only IR gene expression increased under the effect of $1,25(OH)_2D_3$, but also its concentration and activation state. Although this is the first study to report the regulating effect of $1,25(OH)_2D_3$ on IR expression and activity in cultured muscle cells, previous studies have shown that IR mRNA expression is significantly higher in skeletal muscle of rats-fed high calcium and vitamin D diet [26]. Our study confirms that the $1,25(OH)_2D_3$-mediated action on the insulin pathway in muscle cell also involves improved insulin signaling, specifically increasing phosphorylation of Akt/PKB and GSK3. Interestingly, vitamin D deficiency has frequently been associated with insulin resistance in vivo [24], while treatment with vitamin D has been associated with increased expression of the IR in various tissues, including skeletal muscle [25, 26]. In addition, it has been previously shown that exposure to $1,25(OH)_2D_3$ was able to restore de novo protein synthesis triggered by insulin in islets of Langerhans isolated from vitamin D deficient rats [41].

Apart from the regulation of glucose metabolism, insulin together with leucine stimulates protein synthesis in cultured myotubes [33]. Here, we demonstrated for the first time that the protein synthesis activated by insulin and leucine in C12C12 cells was further increased by $1,25(OH)_2D_3$. To the best of our knowledge, only one previous study published by Birge and Haddad in 1975 [37] identified the potential action of a vitamin D precursor, i.e. $25(OH)D_3$, on skeletal muscle. The authors found that intravenous infusion of a high dose of $25(OH)D_3$ in rats increased the in vitro rate of [^{3}H] leucine incorporation into proteins, i.e. a reliable index of protein synthesis rate, in incubated diaphragm or epitrochlearis muscle. This observation alongside our data points to the conclusion that $1,25(OH)_2D_3$ and its immediate precursors (at least $25(OH)D_3$) directly act on muscle protein synthesis, likely through a mechanism involving myotubes, as shown here.

The apparent effect of both $25(OH)D_3$ [37] and $1,25(OH)_2D_3$ (here) on muscle protein metabolism and their mechanisms of action are of obvious interest. Here, the effect of $1,25(OH)_2D_3$ in enhancing the action of insulin and leucine to stimulate muscle protein synthesis was related to the activation of the Akt/PKB and mTOR-mediated pathway. Akt phosphorylates and consequently inhibits GSK3, which subsequently activated eIF2B (eukaryotic translation initiation factor 2B) leading to the formation of the 43S pre-initiation complex [42, 43]. In addition, mTOR phosphorylates 4EBP1 and p70S6K enabling cap-proximal association of the 43S pre-initiation complex. Based on its enhanced effect on the stimulating action of insulin and leucine on this pathway, $1,25(OH)_2D_3$ may well have an additive or potentiating action on the effect of insulin + leucine on muscle cells. This property is likely to be at least partly dependent on the effect of $1,25(OH)_2D_3$ on IR expression and activation. It should also be emphasized that the increase in the transcript and protein levels of VDR and IR receptors was disproportionately higher than the activation of the Akt/PKB and mTOR-mediated pathway, indicating that other regulatory mechanisms are involved. One likely mechanism is a self regulation of the Akt/PKB and mTOR-mediated pathway, especially the existence of a negative feedback loop involving p70 S6 kinase that slows down the activation of the Akt/PKB and mTOR-mediated pathway in stimulated C2C12 myotubes treated with $1,25(OH)_2D_3$ [44]. Future studies will tell.

Despite this observation, a biological action of $1,25(OH)_2D_3$ on amino acid transporters and/or sensor expression and affinity and the related intracellular pathway activation cannot be ruled out. Although additional studies are needed to confirm this hypothesis, there is already a strong rationale for developing research on the putative existence of VDREs in the promoter and/or introns of amino acid transporter genes.

It can be concluded from the present data that $1,25(OH)_2D_3$ has the potential to directly alter protein synthesis in murine C2C12 myotubes. In addition, to the best of our knowledge, this is the first report to identify the effect of $1,25(OH)_2D_3$ on VDR and IR that could account for the anabolic effect of vitamin D on muscle cells. The transcriptional induction of these genes as well as a potentiation of the insulin and leucine action on key related proteins is likely one of the central mechanisms of action of vitamin D on skeletal muscle anabolism. Overall, our data open up perspectives for potentially valuable nutritional interventions coupling vitamin D and amino acid supplementation, mainly in situations like sarcopenia where vitamin D and amino acid response is deficient, to support muscle fiber protein synthesis.

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