

Dose-dependent effects of vitamin D on transdifferentiation of skeletal muscle cells to adipose cells

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Abstract

Fat infiltration within muscle is one of a number of features of vitamin D deficiency, which leads to a decline in muscle functionality. The origin of this fat is unclear, but one possibility is that it forms from myogenic precursor cells present in the muscle, which transdifferentiate into mature adipocytes. The current study examined the effect of the active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), on the capacity of the C2C12 muscle cell line to differentiate towards the myogenic and adipogenic lineages. Cells were cultured in myogenic or adipogenic differentiation media containing increasing concentrations (0, 10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M) of 1,25(OH)₂D₃ for up to 6 days and markers of muscle and fat development were measured. Mature myofibres were formed in both adipogenic and myogenic media, but fat droplets were only observed in adipogenic media. Relative to controls, low physiological concentrations (10⁻¹³ and 10⁻¹¹ M) of 1,25(OH)₂D₃ increased fat droplet accumulation, whereas high physiological (10⁻⁹ M) and supraphysiological concentrations (≥ 10⁻⁷ M) inhibited fat accumulation. This increased accumulation of fat with low physiological concentrations (10⁻¹³ and 10⁻¹¹ M) was associated with a sequential up-regulation of *Pparγ2* (*Pparg*) and *Fabp4* mRNA, indicating formation of adipocytes, whereas higher concentrations (≥ 10⁻⁹ M) reduced all these effects, and the highest concentration (10⁻⁵ M) appeared to have toxic effects. This is the first study to demonstrate dose-dependent effects of 1,25(OH)₂D₃ on the transdifferentiation of muscle cells into adipose cells. Low physiological concentrations (possibly mimicking a deficient state) induced adipogenesis, whereas higher (physiological and supraphysiological) concentrations attenuated this effect.

Key Words

- ▶ myogenesis
- ▶ adipogenesis
- ▶ vitamin D
- ▶ transdifferentiation

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Introduction

Vitamin D (VitD) is a key nutrient for maintaining the health of the musculoskeletal system, with VitD deficiency leading to myopathy, classically characterised by hypotonia, weakness and atrophy of skeletal muscle, and a deterioration in physical capacity (Ceglia 2008). Muscle biopsies from VitD-deficient adults demonstrate enlarged

interfibrillar spaces, fibrosis and loss of type II fibre complement (Yoshikawa *et al.* 1979, Ceglia 2008). There is also an increase in fat infiltration within the muscle (Ceglia 2008, Oh *et al.* 2009, Tagliafico *et al.* 2010) and a similar effect is observed in elderly individuals where the progressive loss in muscle mass and strength seen at the

onset of sarcopenia is associated with an increase in fat deposition within the tissue (Ryall *et al.* 2008).

It is of major concern that VitD deficiency is particularly prevalent amongst the elderly population, as its effects on the musculoskeletal system compound the degenerative effects of sarcopenia (Holick 2007). This can have major consequences for their welfare, as the resultant decline in basic muscle function leads to an increased risk of falls and bone fractures. This deterioration in muscle strength and functionality is thought to result from not just the loss in muscle fibres but also a progressive infiltration of fat within the tissue (Goodpaster *et al.* 2001, Ryall *et al.* 2008). This fat infiltration has been shown to directly impact on muscle strength and functionality and is a key independent risk factor for metabolic diseases such as insulin resistance and diabetes (Goodpaster *et al.* 2003, Zoico *et al.* 2010).

In the blood circulation, VitD is found in two main forms – calcidiol or 25-hydroxyvitamin D (25(OH)D), which is an inactive precursor form, and calcitriol or 1,25-dihydroxyvitamin D (1,25(OH)₂D), which is the active form. Blood concentrations of calcidiol range between 30 and 50 nM (i.e. $3\text{--}5 \times 10^{-8}$ M; McLeod & Cooke 1989), whereas calcitriol is much lower, ranging between ~2 and 350 pM (i.e. 2×10^{-12} – 3.5×10^{-10} M; Zittermann *et al.* 2009). The active form, calcitriol, is formed when circulating 25(OH)D is hydroxylated by the 1 α -hydroxylase enzyme (CYP27b1), present mainly in the kidney, to form the active 1,25(OH)₂D (Takeyama & Kato 2011). Activation then enables 1,25(OH)₂D₃ to bind to the VitD receptor (VDR), a type of nuclear receptor, and thereby regulates transcription of a number of VitD target genes, which is thought to be the principal mechanism of action of VitD. Down-regulation of this response occurs via activation of the 24-hydroxylase enzyme (CYP24a1), which hydroxylates various forms of VitD at carbon 24 resulting in inactivation and targeting for excretion (Holick 2007). *De novo* synthesis of components required for calcium cycling, phospholipid metabolism and cell proliferation/differentiation in muscle is thought to be mediated by VDR-driven mechanisms operating at the level of gene transcription (Drittanti *et al.* 1989, Ceglia 2008), thereby playing an important role in maintaining muscle structure and functionality.

The effects of VitD deficiency are reversible, and studies have shown that VitD supplementation increases the relative number and size of type II fibres in aged skeletal muscle, which can improve balance, increase overall muscle strength and ultimately reduce the incidence of falls (Bischoff *et al.* 2003, Harwood *et al.* 2004,

Sato *et al.* 2005, Moreira-Pfrimer *et al.* 2009). However, it is not known what effect VitD supplementation has on fat infiltration within muscle. Indeed, the origin of these adipose cells and the mechanism by which they mature within muscle remains unclear. It may correspond to aberrant transdifferentiation of myogenic precursor cells into adipocytes resulting in the formation of fat within the intermuscular space (Vettor *et al.* 2009). Certainly numerous studies have demonstrated that myogenic precursor cells retain the potential to transdifferentiate towards the adipogenic lineage (Hu *et al.* 1995, Grimaldi *et al.* 1997, Holst *et al.* 2003, Seale *et al.* 2008, Vettor *et al.* 2009). Previous work has shown that VitD has potent effects on both adipogenesis (Ishida *et al.* 1988, Sato & Hiragun 1988, Lenoir *et al.* 1996, Blumberg *et al.* 2006, Kong & Li 2006, Thomson *et al.* 2007, Zhuang *et al.* 2007) and myogenesis (Capiati *et al.* 1999, Garcia *et al.* 2011). Most have used physiologically relevant concentrations of 1,25(OH)₂D₃, but some have tended to use high physiological or supraphysiological concentrations. Importantly, it is not known whether VitD affects the transdifferentiation of myogenic precursor cells into adipocytes. In order to address this question, the current study investigated the effect of a broad range of concentrations of the active form of VitD₃ (1,25(OH)₂D₃) on the capacity of the murine C2C12 muscle cell line to differentiate or transdifferentiate towards the myogenic or adipogenic lineages respectively. We included 1,25(OH)₂D₃ concentrations covering the physiological range (10^{-13} – 10^{-9} M), as well as the supraphysiological concentrations (10^{-7} and 10^{-5} M) used previously in other cell culture studies (Blumberg *et al.* 2006, Zhuang *et al.* 2007, Garcia *et al.* 2011).

Materials and methods

Cell culture and reagents

C2C12 cells were cultured in six-well plates in growth media consisting of DMEM (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (hi-FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin and maintained at 37 °C under 5% CO₂ until 70–80% confluent. This corresponded to day 0 of differentiation, at which stage cells were switched to either myogenic or adipogenic differentiation media and incubated for up to 6 days at 37 °C under 5% CO₂. Myogenic differentiation media consisted of DMEM containing 2% horse serum and antibiotics and was changed every 2 days until the end of the experiment. For induction of adipogenic differentiation, cells were

cultured in DMEM containing 10% hi-FBS, antibiotics, 0.5 mM isobutylmethylxanthine, 1 μ M dexamethasone, 850 nM insulin, 10 nM tri-iodothyronine (T_3) and 1 μ M rosiglitazone (peroxisome proliferator activated receptor γ (PPAR γ) agonist) from days 0 to 2. This induction medium was then replaced with DMEM containing 10% hi-FBS, antibiotics, 850 nM insulin, 10 nM T_3 and 1 μ M rosiglitazone from day 2 onwards and changed every 2 days until the end of the experiment. Both myogenic and adipogenic differentiation media were supplemented with increasing concentrations (0, 10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} or 10^{-5} M) of 1,25(OH) $_2$ D $_3$ (Sigma), which was dissolved and diluted in DMSO. All treatments (including control) contained 0.1% (v/v) DMSO.

Oil Red-O and haematoxylin staining

Accumulation of lipid droplets was monitored by phase contrast microscopy. After 6 days of exposure to myogenic or adipogenic differentiation media, cells were stained with Oil Red-O to identify lipid droplets and counter-stained with haematoxylin to delineate nuclear and myofibre structures. Briefly, media were removed and cells were fixed in 3.7% formaldehyde at room temperature for 30 min. Cells were washed twice with pre-warmed (to 37 °C) PBS and then with 60% isopropanol, before staining with 0.3% Oil Red-O (Sigma) for 30 min at room temperature. Cells were then washed once in 60% isopropanol and twice in tap water, before counter-staining with Harris haematoxylin (Sigma) for 3 min, all at room temperature. Excess stain was removed by washing twice in distilled water and then another two times in Scott's tap water (0.24 M sodium bicarbonate and 0.03 M magnesium sulphate). Images were captured using an Olympus SZH10 microscope (Olympus, Southend-on-Sea, UK) and analysed using Image Pro (version 5.1, Rockville, MD, USA) for the quantification of Oil Red-O staining. The Image Pro Software detected the Oil Red-O staining using a fixed threshold and then calculated percentage area of staining for each image. Five field of view images from each of four replicate wells (i.e. 20 in total) were quantified for each treatment.

Quantitative real-time PCR analysis

At the required timepoints (days 0, 2, 4 and 6), total RNA was extracted from C2C12 cells using a High Pure RNA isolation kit (Roche), according to the manufacturer's protocol. Samples were then stored frozen (-80 °C) before first-strand cDNA synthesis using random hexamer

primers, as described previously (Hemmings *et al.* 2009). Quantitative PCR was performed in duplicate using SYBR1 Master mix (Roche), 384-well microplates and the LightCycler LC480 (Roche) configured for SYBR green detection, again as described previously (Tonge *et al.* 2010). The primers for each transcript were designed using Primer Express (Applied Biosystems) and are shown in Table 1. A standard curve was produced using serial dilutions of a pool of cDNA made from all samples to check the linearity and efficiency of the PCR for every gene, while melt curve analysis was used to check whether there was only a single product. Transcript abundance was determined using the standard curve method as described previously (Brown *et al.* 2012). Previous studies in this cell line (Brown *et al.* 2012) had demonstrated that expression of all the common 'housekeeper' genes changes during differentiation; therefore, all data were normalised to total first-strand cDNA content, measured using Oligreen, as described previously (Brown *et al.* 2012).

Creatine kinase, DNA and protein assays

At the required timepoint (day 4), C2C12 cells from each well were scraped into 1 ml of cold (4 °C) tri-sodium citrate buffer (0.05 M, pH 6.8) and sonicated on ice for 15 s using a benchtop ultrasonicator (Soniprep 150; MSE, London, (UK) Ltd.), being stored frozen (-80 °C) before analysis. Creatine kinase (CK) activity (IU/well) was measured using a CK assay kit (Thermo Scientific Cramlington, Northumberland, UK), as described previously (Brown *et al.* 2012). Briefly, thawed samples were transferred on to a 96-well microtitre plate and 200 μ l of reaction buffer was added according to the manufacturer's instructions. Absorption at 340 nm was measured at 30 °C every 5 min over a 30-min period. DNA content (μ g/well) of the thawed cell lysate was measured using a fluorescence plate reader assay adapted from Rago *et al.* (Rago *et al.* 1990, Hurley *et al.* 2006). Protein content (μ g/well) was measured via the Lowry method (Lowry *et al.* 1951) adapted for 96-well plate format. Usually, we normalise the CK activity data to DNA content to account for differences in numbers of cells in each well. However, in this case, normalising the CK activity data to DNA resulted in the differences in CK activity observed between media types (myogenic vs adipogenic) being exaggerated due to the obvious differences in DNA contents (i.e. numbers of cells and their differing morphology), as there were clear differences in the types of cells present. We therefore opted to include the three measurements (CK activity, protein and DNA) separately on a per well basis.

Table 1 Mouse-specific Primer sequences used for quantitative real-time PCR analysis. Primer sequences for PPAR γ 1, PPAR γ 2 and UCP1 were obtained from Kajimura *et al.* (2009)

Gene	Primer sequence (5'–3')
<i>MyoD</i>	Forward: CGTGGCAGCGAGCACTAC Reverse: TGTAATCCATCATGCCATCAGA
Creatine kinase	Forward: GCACTGGCCGAGCAT Reverse: GAGGGTAGTACTTGCCCTTGAAGCTC
Myogenin	Forward: CCCATGGTCCCAGTGAA Reverse: GCAGATTGTGGGCGTCTGTA
<i>Myf5</i>	Forward: GACCCCCACCTCCAAGT Reverse: CAGCACATGCATTGATACATC
<i>Pparγ1</i>	Forward: TGAAAGAAGCGGTGAACCACTG Reverse: TGGCATCTCTGTGCAACCATG
<i>Pparγ2</i>	Forward: GCATGGTGCCTTCGCTGA Reverse: TGGCATCTCTGTGCAACCATG
<i>Fabp4</i>	Forward: AAGTGGGAGTGGGCTTTGC Reverse: TGGTGACCAAATCCCCATT
<i>AdipoQ</i>	Forward: GACACAAAAGGGCTCAGGAT Reverse: TGGGCAGGATTAAGAGGAACA
Leptin	Forward: CAAGCAGTGCCTATCCAGAAAAGT Reverse: CAAGCAGTGCCTATCCAGAAAAGT
<i>Ucp1</i>	Forward: ACTGCCACACTCCAGTCATT Reverse: CTTTGCCTCACTCAGGATTGG
<i>Prdm16</i>	Forward: GAAGGTGTCCAACTGACAATGC Reverse: CGTCACTTTTGCTAGCTTCCT
<i>Elovl3</i>	Forward: ATGAATTTCTCACGGGGTTA Reverse: GCTTACCCAGTACTCCTCCAAAAA
<i>Cidea</i>	Forward: CCAGAGTCACCTTCGACCTATAACA Reverse: CTCGTACATCGTGGCTTTGACA
<i>Clebpβ</i>	Forward: AGCGGCTGCAGAAGAAGGT Reverse: GGCAGTGTCTGAACAAGTTC
<i>Pgc1α</i>	Forward: TGCAGCGGTCTTAGCACTCA Reverse: CATGAATTCTCGGTCTTAACAATGG

Statistical analysis

Data were analysed by one- or two-way ANOVA for effects of 1,25(OH) $_2$ D $_3$ concentration, media type (myogenic or adipogenic) and/or timepoint (as appropriate) using SPSS Statistical Software (19th Edition, Portsmouth, Hampshire, UK). Percentage area of Oil Red-O staining was analysed by one-way ANOVA (1,25(OH) $_2$ D $_3$ concentration only). CK activities, DNA and protein contents were analysed by two-way ANOVA (1,25(OH) $_2$ D $_3$ concentration and media type). All real-time qPCR data were analysed by two-way ANOVA (1,25(OH) $_2$ D $_3$ concentration and timepoint), with the data from myogenic and adipogenic media being analysed separately. A *post-hoc* Bonferroni's test was used when appropriate (i.e. no significant interactions). All means are for either $n=3$ (CK activity, protein and DNA contents) or $n=4$ (Oil Red-O staining and all mRNA expression) replicates (i.e. wells) for each treatment and media type at each timepoint. $P<0.05$ was considered statistically significant.

Results

Effects of 1,25(OH) $_2$ D $_3$ on myotube formation and lipid accumulation

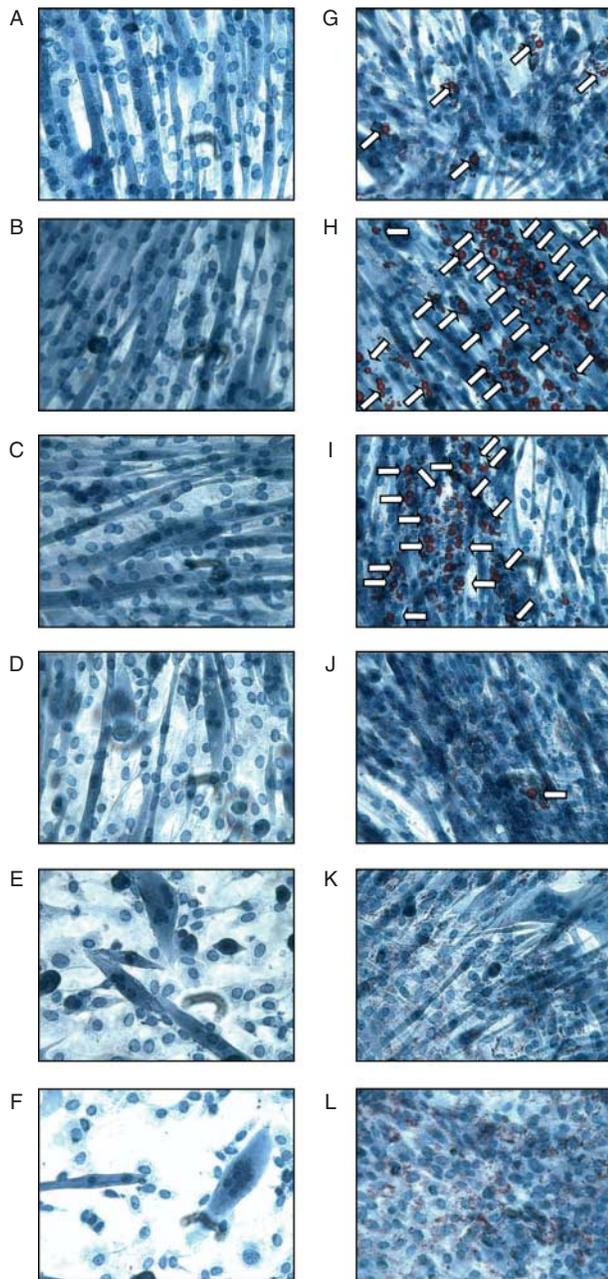
Myotube formation was clearly evident in control cells after 6 days in either myogenic or adipogenic differentiation media (Fig. 1A and G). Phase contrast images of control cells showed extensive myotube formation by day 4 in myogenic media, which did not appear to increase any further on day 5 (data not shown). By contrast, myotubes were still visibly forming throughout days 4 and 5 of differentiation in control cells in adipogenic media (data not shown), suggesting that myogenic differentiation was slightly delayed, but there appeared to be no difference in myotube formation between myogenic and adipogenic media on day 6 (Fig. 1A and G).

Positive Oil Red-O staining confirmed the presence of lipid droplets in control cells grown for 6 days in adipogenic media (Fig. 1G), but there was no Oil Red-O staining of cells grown in myogenic media (either without or with 1,25(OH) $_2$ D $_3$) for the same length of time (Fig. 1A, B, C, D, E and F). Similarly, phase contrast images taken at earlier timepoints demonstrated the formation of lipid droplets after 4 days, but only in cells cultured in adipogenic media (data not shown).

Dose-dependent effects of 1,25(OH) $_2$ D $_3$ supplementation for 6 days were observed on myotube formation, with addition of supraphysiological concentrations (10^{-7} and 10^{-5} M) appearing to inhibit myotube formation in both myogenic and adipogenic media (Fig. 1E, F, K and L). Relative to the control cells grown for 6 days in adipogenic media, supplementation with increasing concentrations of 1,25(OH) $_2$ D $_3$ induced a bimodal effect on lipid droplet accumulation in cells cultured in adipogenic media, as determined by percentage area of Oil Red-O staining (Fig. 2). At the lowest physiological concentration (10^{-13} M), 1,25(OH) $_2$ D $_3$ significantly increased lipid droplet accumulation compared with controls ($P<0.01$ Bonferroni; Figs 1H and 2), whereas the highest concentrations (10^{-9} M and above), corresponding to high physiological and supraphysiological concentrations, inhibited lipid droplet formation ($P<0.001$ Bonferroni; Figs 1J, K, L and 2).

Expression of gene markers of white adipocytes

As observed for lipid droplet formation (Fig. 1G, H, I, J, K and L), C2C12 cells grown in adipogenic media demonstrated induction of genes indicative of differentiation of

**Figure 1**

Dose-dependent effects of $1,25(\text{OH})_2\text{D}_3$ on accumulation of lipid droplets in C2C12 cells cultured in myogenic (A, B, C, D, E and F) or adipogenic (G, H, I, J, K and L) media. Representative images of C2C12 cells stained with Oil Red-O (as indicated by white arrows) and counterstained with haematoxylin to indicate lipid droplet and nuclei/myofibre structures respectively. Cells were cultured for 6 days in myogenic (A, B, C, D, E and F) or adipogenic (G, H, I, J, K and L) media with vehicle (A and G) or increasing concentrations of $1,25(\text{OH})_2\text{D}_3$ (10^{-13} M (B and H), 10^{-11} M (C and I), 10^{-9} M (D and J), 10^{-7} M (E and K) or 10^{-5} M (F and L)).

white adipocytes (Fig. 3A, B and C). For clarity, we only include figures for the expression of genes in adipogenic media, but the figures showing expression of these same genes by cells cultured in myogenic media are included in the Supplementary Figure 1A, B, C and D, see section on supplementary data given at the end of this article. In adipogenic media, expression of all three adipose-specific marker genes, *Ppar γ 2* (*Pparg*; Fig. 3A), fatty acid binding protein 4 (*Fabp4*; Fig. 3B) and adiponectin/*Adipoq* (Fig. 3C), were induced in a $1,25(\text{OH})_2\text{D}_3$ concentration and time-dependent manner ($P < 0.001$ for all three $1,25(\text{OH})_2\text{D}_3$ concentration \times day interactions), but only in cells grown in adipogenic media. Low physiological concentrations (10^{-13} and 10^{-11} M) of $1,25(\text{OH})_2\text{D}_3$ increased expression of all three genes, with levels of PPAR γ 2 peaking at day 2 and declining over days 4 and 6 (Fig. 3A), while expression of the PPAR γ 2 target genes, *Fabp4* and *Adipoq*, peaked 2 days later at day 4 of differentiation (Fig. 3B and C). Interestingly, the high physiological (10^{-9} M) and supraphysiological (10^{-7} and 10^{-5} M) concentrations of $1,25(\text{OH})_2\text{D}_3$ decreased expression of all three genes, corresponding to the observed decrease in lipid droplet formation. By contrast, expression of these genes by cells incubated in myogenic media was either undetectable (PPAR γ 2; Supplementary Figure 1A) or only expressed in the presence of supra-physiological concentrations of $1,25(\text{OH})_2\text{D}_3$ (FABP4 and ADIPOQ; Supplementary Figure 1B and C). Leptin mRNA was not detectable in any of the cultures (in adipogenic or myogenic media) throughout the 6-day period (data not shown). In contrast to PPAR γ 2, which is known to be adipocyte specific, *Ppar γ 1* mRNA was detected in all cultures (in both myogenic and adipogenic media) and was found to be higher at day 2 than at day 6 ($P = 0.002$ for day effect; Fig. 3D) in adipogenic media and higher in controls and 10^{-5} M $1,25(\text{OH})_2\text{D}_3$ than the other concentrations ($P = 0.005$ for $1,25(\text{OH})_2\text{D}_3$ concentration effect; Fig. 3D) again in adipogenic media.

Effects of $1,25(\text{OH})_2\text{D}_3$ on myogenic differentiation in myogenic and adipogenic media

It was apparent from the morphological studies that supplementation with supraphysiological concentrations (10^{-7} and 10^{-5} M) of $1,25(\text{OH})_2\text{D}_3$ inhibited myotube formation in both myogenic and adipogenic media (Fig. 1E, F, K and L). We therefore determined CK activities after 4 days of treatment, as a quantitative measure of myogenic differentiation. Lower CK activities were observed for control cells in adipogenic compared with

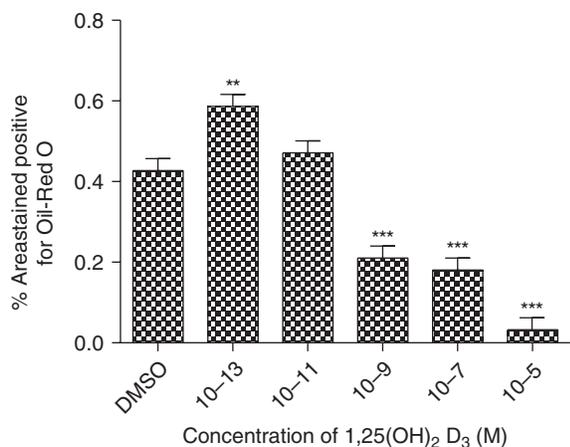


Figure 2

Dose-dependent effects of 1,25(OH)₂D₃ on percentage area of lipid accumulation in C2C12 cells cultured in adipogenic media for 6 days. C2C12 cells were stained with Oil Red-O and counterstained with haematoxylin, before the percentage area of the red stain within a field of view (FOV) was quantified by image analysis to give a representative percentage area per well ($n=4$ wells, five FOV/well). One-way ANOVA indicated a significant effect of 1,25(OH)₂D₃ ($P<0.001$). Significant differences compared to DMSO control cells were determined by *post-hoc* Bonferroni's test (** $P<0.01$ and *** $P<0.001$).

myogenic media (Fig. 4A), but supplementation of the adipogenic media with 1,25(OH)₂D₃ increased CK activity to levels approaching those observed in control cells in myogenic media and the magnitude of this increase was similar between 10⁻¹³ and 10⁻⁷ M ($P<0.001$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4A). By contrast, cells exposed to 10⁻⁵ M 1,25(OH)₂D₃ in adipogenic media showed a decrease in CK activity compared with controls ($P<0.001$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4A), indicating an attenuation of myogenic differentiation, consistent with the observed absence of myotubes in these cultures (Fig. 1L). In myogenic media, addition of 1,25(OH)₂D₃ at 10⁻⁷ and 10⁻⁵ M was also associated with a reduction in CK activity ($P<0.001$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4A), as well as reductions in total protein ($P<0.01$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4B) and total DNA ($P<0.001$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4C) contents at day 4. Protein (Fig. 4B) and DNA (Fig. 4C) contents were greater in cells cultured in adipogenic compared with myogenic media, indicative of increased cell proliferation, probably due to the higher FBS content of the adipogenic media. Similar to effects in myogenic media, increasing 1,25(OH)₂D₃ concentrations in adipogenic

media decreased both protein ($P<0.01$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4B) and DNA ($P<0.001$ for media \times 1,25(OH)₂D₃ concentration interaction; Fig. 4C) contents in a dose-dependent manner, but particularly at the highest (supraphysiological) concentrations.

Expression of myogenic marker genes

Consistent with myotube formation being observed in both types of differentiation media (Fig. 1A, B, C, D, E, F, G and H), muscle-specific genes were expressed in cells grown in either myogenic or adipogenic media. For clarity, we will mainly describe the results from studies using adipogenic media here, but figures for expression of the same genes by cells cultured in myogenic media are included in the [Supplementary Figure 1E, F, G and H](#). The effects of 1,25(OH)₂D₃ (particularly physiological concentrations) on myogenic marker genes were more pronounced in adipogenic media (Fig. 5A, B, C and D) compared with myogenic media ([Supplementary Figure 1E, F, G and H](#)), consistent with the results obtained for CK activity. Similar to CK activity, CK mRNA expression was lower in adipogenic media compared with myogenic media (Fig. 5A and [Supplementary Figure 1E](#)). An increase in CK mRNA was observed at day 4 in control cells (in adipogenic media), but supplementation of adipogenic media with physiological concentrations (10⁻¹³, 10⁻¹¹ and 10⁻⁹ M) of 1,25(OH)₂D₃ increased CK mRNA expression particularly on day 4, whereas 10⁻⁵ M 1,25(OH)₂D₃ blocked/inhibited differentiation at all time-points ($P<0.001$ for 1,25(OH)₂D₃ concentration \times day interaction; Fig. 5A). A similar pattern was observed for myogenin expression (Fig. 5B). Adipogenic media induced a smaller increase in myogenin mRNA on day 2 compared with myogenic media (Fig. 5B and [Supplementary Figure 1F](#)), but myogenin mRNA continued to increase on days 4 and 6 in adipogenic media and actually exceeded the levels observed in myogenic media (Fig. 5B and [Supplementary Figure 1F](#)). Supplementation of adipogenic media with 10⁻¹³, 10⁻¹¹, 10⁻⁹ or 10⁻⁷ M 1,25(OH)₂D₃ increased myogenin mRNA, particularly on day 2, whereas 10⁻⁵ M 1,25(OH)₂D₃ blocked differentiation at all timepoints ($P<0.001$ for 1,25(OH)₂D₃ concentration \times day interaction; Fig. 5B). In adipogenic media, there was no change in *Myod1* (*MyoD*) expression in control cells throughout the 6 days of differentiation (Fig. 5C), but supplementation with 10⁻¹³, 10⁻¹¹, 10⁻⁹ or 10⁻⁷ M 1,25(OH)₂D₃ increased *MyoD* on days 2 and 4, whereas 10⁻⁵ M 1,25(OH)₂D₃ blocked these effects ($P=0.001$ for

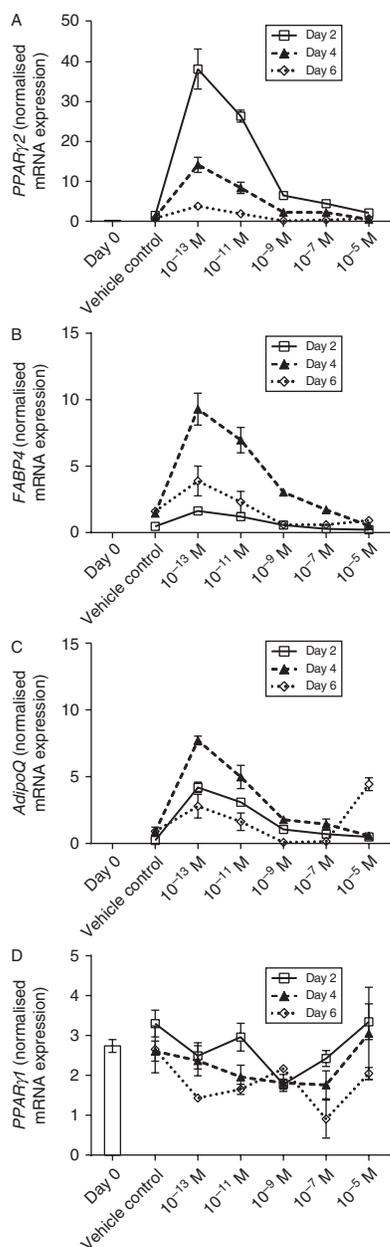


Figure 3

Dose-dependent effects of 1,25(OH)₂D₃ on expression of white adipocyte marker genes. Expression of white adipocyte marker genes was determined by quantitative RT-PCR analysis. Levels of (A) *Ppar γ 2*, (B) *Fabp4*, (C) *Adipoq*/adiponectin and (D) *Ppar γ 1* mRNAs were quantified in C2C12 cells cultured in the absence or presence of 10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M 1,25(OH)₂D₃ for 2, 4 or 6 days in adipogenic differentiation media. Expression at day 0 (before differentiation media and 1,25(OH)₂D₃ was added) is also included and is indicated by a bar (in some instances, this was very low). Significant two-way interactions between day of differentiation and 1,25(OH)₂D₃ concentration were observed for PPAR γ 2, FABP4 and AdipoQ ($P < 0.001$ for all). For PPAR γ 1, there was a significant effect of stage of differentiation ($P = 0.002$) and a significant effect of 1,25(OH)₂D₃ concentration ($P = 0.005$), but no interaction.

1,25(OH)₂D₃ concentration \times day interaction; Fig. 5C). Finally, *myf5* mRNA was up-regulated in control cells in adipogenic media at days 2 and 4 (Fig. 5D), but this was inhibited by increasing concentrations of 1,25(OH)₂D₃ in a dose-dependent manner, with 10⁻⁵ M 1,25(OH)₂D₃ appearing to block differentiation ($P < 0.001$ for 1,25(OH)₂D₃ concentration \times day interaction; Fig. 5D).

In summary, there were only relatively small effects of 1,25(OH)₂D₃ on cells incubated in myogenic media (see Supplementary Figure 1E, F, G and H), mainly due to the effects of the highest (supraphysiological) concentration (10⁻⁵ M). However, supplementation of adipogenic media with physiological (10⁻¹³, 10⁻¹¹ and 10⁻⁹ M) concentrations of 1,25(OH)₂D₃ increased CK, *MyoD* and myogenin mRNA, suggesting an induction of muscle differentiation. Importantly, 10⁻⁵ M 1,25(OH)₂D₃ blocked/inhibited expression of all four myogenic marker genes in adipogenic media, suggesting that this concentration (10⁻⁵ M) may have a different effect to the other concentrations, possibly involving anti-differentiation, pro-apoptotic and/or toxic effects.

Expression of gene markers of brown adipocytes

As 1,25(OH)₂D₃ was shown to induce gene markers of white adipocytes, we also considered its effect on activation of genes relating to brown adipocytes. Once again, for clarity, only the data from studies in adipogenic media are included, but data for myogenic media are provided in the Supplementary Figure 2A, B, C and D, see section on supplementary data given at the end of this article. Expression of the brown fat-specific marker, uncoupling protein 1 (UCP1), was below detectable levels in cells grown in either myogenic or adipogenic media (data not shown). Likewise, expression of PRD1-BF1-RIZ1 homologous domain containing 16 (*Prdm16*), previously shown to be required for the transdifferentiation of C2C12 cells to brown adipocytes (Seale *et al.* 2008), was also below detectable limits (data not shown). However, expression of other brown fat marker genes, *Elovl3* and *CIDEA*, were detectable in cells cultured in adipogenic media, but only after 6 days ($P < 0.001$ for day effect for both genes; Fig. 6A and B respectively). This was preceded by a slight decrease in the expression of CCAAT enhancer binding protein β (*C/ebp β*) mRNA ($P < 0.001$ for day effect; Fig. 6C) at days 2–6, but no change in expression of PPAR γ coactivator 1 α (*PGC1 α* ; Fig. 6D) mRNA. In contrast to the changes observed in expression of the white adipogenic marker genes, treatment with 1,25(OH)₂D₃ had no significant effect ($P > 0.05$) on expression of any of

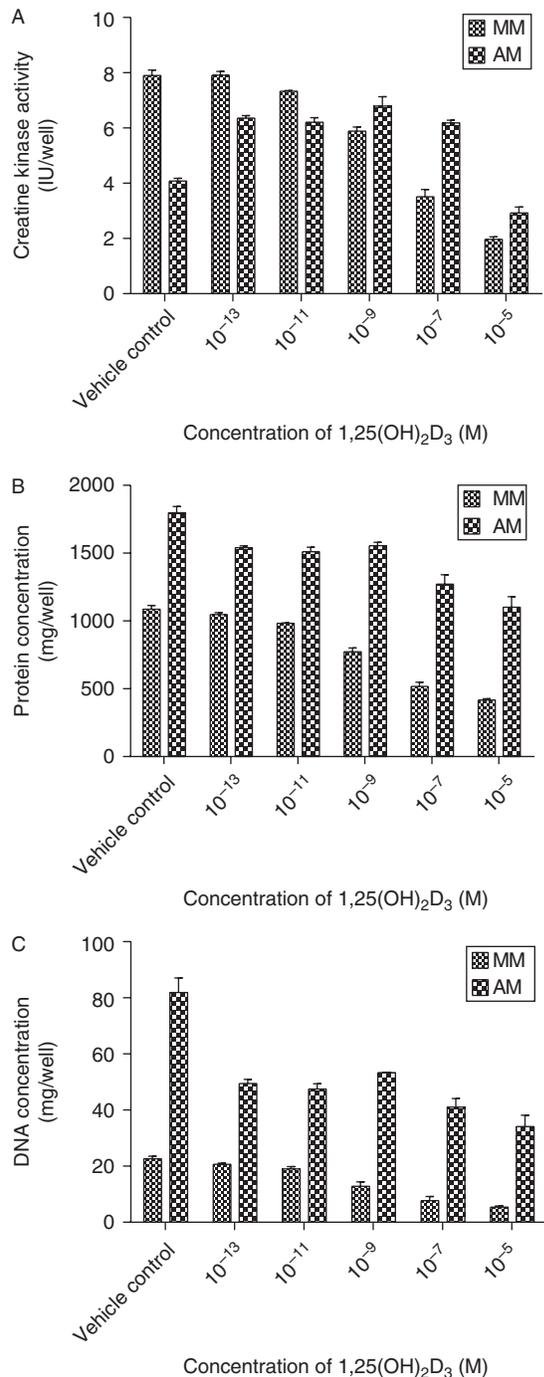


Figure 4 Dose-dependent effects of 1,25(OH)₂D₃ on (A) CK activity (IU/well), (B) protein (μg/well) and (C) DNA content (μg/well) in C2C12 cells cultured for 4 days in myogenic media (MM) or adipogenic media (AM) with increasing concentrations (vehicle control, 10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷ and 10⁻⁵ M) of 1,25(OH)₂D₃. Significant two-way interactions between media type and 1,25(OH)₂D₃ concentration were observed for CK activity ($P < 0.001$), protein ($P < 0.01$) and DNA ($P < 0.001$) content.

the brown adipocyte marker genes, suggesting that 1,25(OH)₂D₃ treatment was not inducing conversion of myoblasts to brown adipocytes, although a longer time frame may be required to be completely sure.

Expression of VDR and the VitD hydroxylating enzymes 1α-hydroxylase and 24-hydroxylase

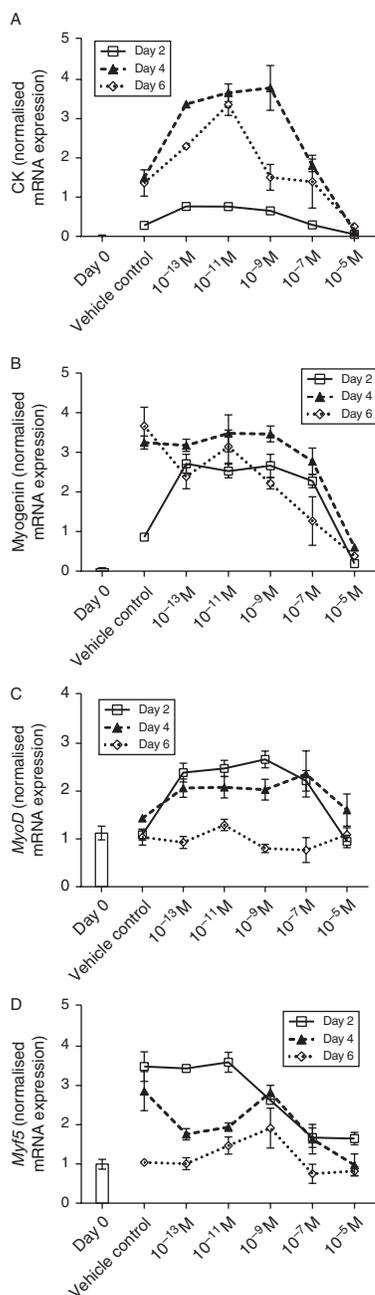
As the activity of the VitD system is dependent on the levels of VDR and metabolising enzymes, we also determined their expression in the cell cultures. Once again, for clarity, the data from adipogenic media will mainly be described here, but the data from myogenic media are provided in the Supplementary Figure 2E, F and G. VDR mRNA was expressed in the C2C12 cells and there was no difference in the level of expression between control cells incubated in either myogenic or adipogenic media (Fig. 7A and Supplementary Figure 2E). In adipogenic media, VDR expression was increased by the highest concentration (10⁻⁵ M) of 1,25(OH)₂D₃ particularly on day 2 ($P < 0.001$ for VitD concentration × day interaction; Fig. 7A). Basal levels of expression of the 25(OH)D₃ activating enzyme, CYP27B1 (1α-hydroxylase) mRNA, increased in control cells incubated in adipogenic media throughout the 6 days of differentiation ($P < 0.001$ for day effect; Fig. 7B), but there was no significant effect of 1,25(OH)₂D₃ on CYP27B1 mRNA expression ($P > 0.05$; Fig. 7B).

In contrast to the activating enzyme, expression of the VitD inactivating enzyme, CYP24a1 (also called 24-hydroxylase), was not detectable in control cells incubated in adipogenic media and did not change with day/stage of differentiation (Fig. 7C), but treatment with 10⁻⁵ M 1,25(OH)₂D₃ induced CYP24A1 mRNA expression ($P < 0.001$ for 1,25(OH)₂D₃ concentration effect; Fig. 7C). Hence, the cells appear to be responding to the very high levels of active VitD by increasing the levels of this inactivating enzyme to avoid or minimise potential toxicity effects.

Discussion

Adipogenic induction

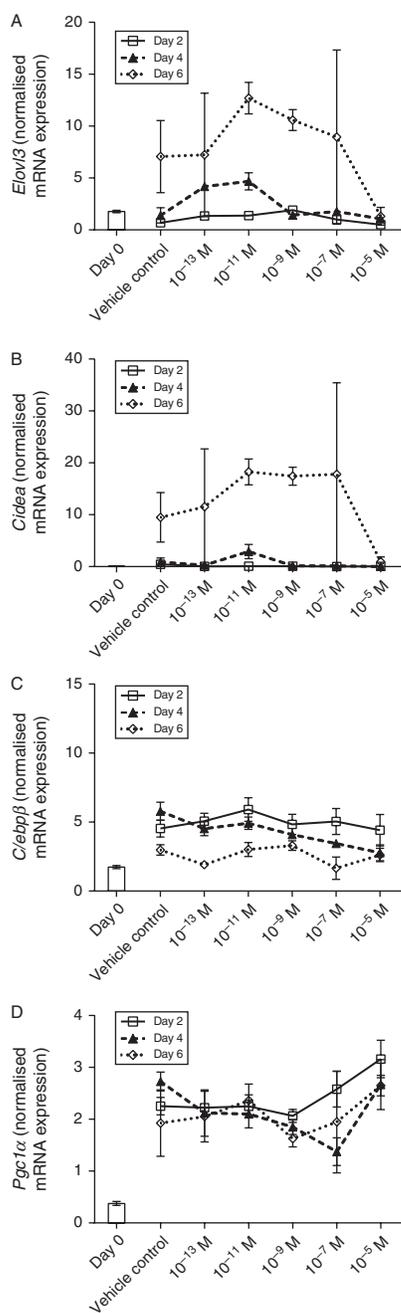
For the first time, this study shows a bimodal dose–response effect of the active form of VitD₃, 1,25(OH)₂D₃, to modulate the capacity of C2C12 cells to transdifferentiate into adipocytes. The adipogenic potential of C2C12 cells has been shown previously, with exposure to thiazolidinediones and fatty acids found to induce transdifferentiation to mature adipocytes

**Figure 5**

Dose-dependent effects of 1,25(OH)₂D₃ on expression of skeletal muscle marker genes. Expression of skeletal muscle marker genes was determined by quantitative RT-PCR analysis. Levels of (A) CK, (B) myogenin, (C) *MyoD* and (D) *Myf5* mRNAs were quantified in C2C12 cells cultured in the absence or presence of 10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M 1,25(OH)₂D₃ for 2, 4 or 6 days in adipogenic differentiation media. Expression at day 0 (before differentiation media and 1,25(OH)₂D₃ was added) is also included for reference and is indicated by a bar. Significant two-way interactions between day of differentiation and 1,25(OH)₂D₃ concentration were observed for CK, myogenin, *Myf5* ($P < 0.001$ for all) and *MyoD* ($P < 0.05$) mRNA transcripts.

(Teboul *et al.* 1995). A key finding from these studies was that physiologically relevant, sub-nanomolar (10⁻¹³ M) concentrations of 1,25(OH)₂D₃ potentially induced accumulation of lipid droplets in adipogenic media. Importantly, this was preceded by a clear up-regulation in expression of the adipogenic marker genes, PPAR γ 2 and FABP4, which were undetectable in cells grown in myogenic media. PPAR γ 2 has previously been shown to be pivotal in inducing the transdifferentiation of myogenic precursor cells to the adipogenic lineage (Hu *et al.* 1995, Yu *et al.* 2006). This study showed that expression of *Ppar γ 2* mRNA peaked on day 2 of differentiation followed by a sequential up-regulation in expression of downstream target genes, *Fabp4* and *Adipoq*, both of which peaked at day 4 in adipogenic media. Interestingly, decreases in lipid accumulation (Oil Red-O percentage area) and adipocyte marker gene expression (*Ppar γ 2* and *Fabp4*) were observed at higher physiological concentrations (between 10⁻¹³ and 10⁻⁹ M) of 1,25(OH)₂D₃ without any change in DNA or protein contents, whereas supraphysiological concentrations (10⁻⁷ and 10⁻⁵ M) of 1,25(OH)₂D₃ completely inhibited lipid droplet accumulation and decreased expression of adipogenic marker genes but also reduced DNA and protein contents. Hence, the mechanisms for the anti-differentiation effects of the highest concentrations are potentially due to anti-proliferative and/or pro-apoptotic/toxic effects, but this appears not to be the case for the lower, more physiological concentrations. Therefore, the bimodal effects of 1,25(OH)₂D₃ in altering the transdifferentiation to an adipogenic lineage occurred over a physiologically relevant concentration range and appeared not to be due to anti-proliferative or toxic effects, suggesting that our findings may be indicative of effects *in vivo*.

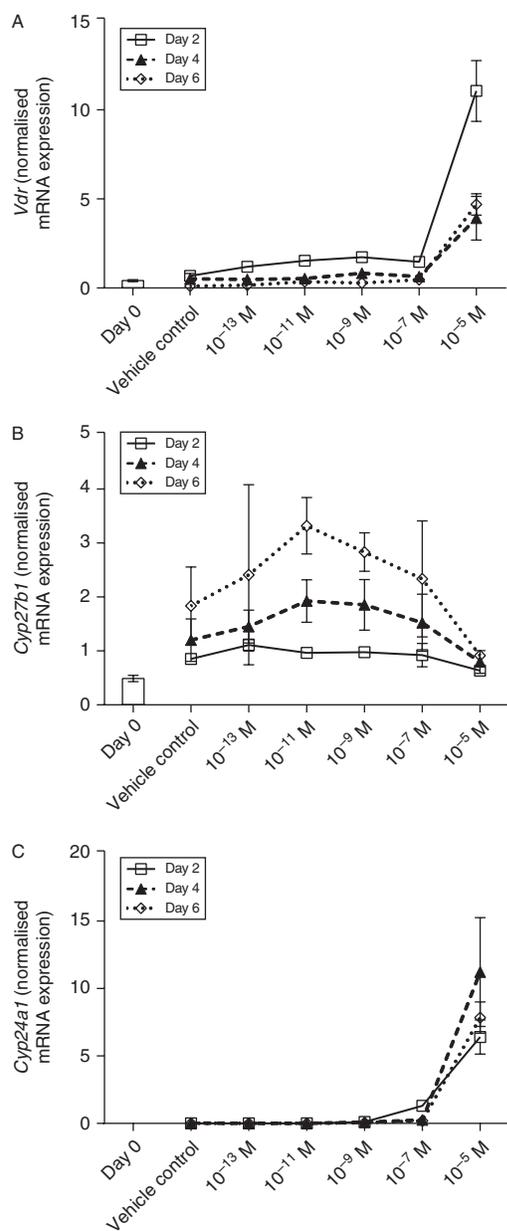
Previous studies have reported similar bimodal effects of 1,25(OH)₂D₃ over similar concentration ranges on adipogenesis in 3T3-L1 (Thomson *et al.* 2007) and Ob17 preadipocytes (Lenoir *et al.* 1996). Consistent with our findings, Lenoir *et al.* (1996) showed that 1,25(OH)₂D₃ stimulated adipogenesis at concentrations of 0.25 nM (2.5 \times 10⁻¹⁰ M) or less, whereas nanomolar concentrations almost completely blocked adipogenic differentiation. Similarly, 0.1 nM (10⁻¹⁰ M) 1,25(OH)₂D₃ stimulated adipogenesis, whereas 1 and 10 nM (10⁻⁹ and 10⁻⁸ M) inhibited adipogenesis in 3T3-L1 preadipocytes (Thomson *et al.* 2007). Inhibitory effects of high nanomolar concentrations (1–100 nM or 10⁻⁹–10⁻⁷ M) of 1,25(OH)₂D₃ on adipogenesis have also been reported in 3T3-L1 preadipocytes (Blumberg *et al.* 2006, Kong & Li

**Figure 6**

Dose-dependent effects of 1,25(OH)₂D₃ on expression of brown adipocyte marker genes. Expression of brown adipocyte marker genes was determined by quantitative RT-PCR analysis. Levels of (A) *Elov13*, (B) *Cidea*, (C) *Clebpβ* and (D) *Pgc1α* mRNAs were quantified in C2C12 cells cultured in the absence or presence of 10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M 1,25(OH)₂D₃ for 2, 4 or 6 days in adipogenic differentiation media. Expression at day 0 (before differentiation media and 1,25(OH)₂D₃ was added) is also included for reference and is indicated by a bar. Significant effects of day of differentiation were observed for *Elov13*, *Cidea* and *Clebpβ* ($P < 0.001$ for all three), but not *PGC1α* ($P > 0.05$). There were no significant effects of 1,25(OH)₂D₃ concentration on any of the brown adipocyte marker genes.

2006, Thomson *et al.* 2007) and were associated with decreases in *C/ebpα* and *Pparγ2* mRNA expression (Blumberg *et al.* 2006, Kong & Li 2006, Thomson *et al.* 2007). Interestingly, 3T3-L1 preadipocytes were only receptive to this inhibitory effect in the early stages of differentiation (i.e. the induction phase), with no effect observed when 1,25(OH)₂D₃ was administered from 48 h onwards (Kong & Li 2006). This receptive period in 3T3-L1 cells may relate to temporal changes in VDR expression, which is rapidly up-regulated in preadipocytes in the first 4–8 h of differentiation and then progressively down-regulated in the following 48 h (Kong & Li 2006). We observed something similar, with higher VDR expression at day 2 compared with days 4 and 6 in adipogenic media. Interestingly, only the highest supra-physiological (10⁻⁵ M) concentration of 1,25(OH)₂D₃ increased expression of VDR, particularly at day 2 of differentiation in adipogenic media.

Having established that C2C12 cells appeared to be induced to transdifferentiate into adipocytes, a number of brown fat-specific marker genes were also measured to clarify whether the cells being formed were white or brown adipocytes. Recent findings from lineage tracing studies have shown that brown adipocytes develop *in vivo* from a MYF5-positive progenitor cell (Seale *et al.* 2008), suggesting that these myf5-expressing C2C12 cells might also be converting to brown adipocytes. Previous work showed that ectopic overexpression of PRDM16 in C2C12 cells induced *Myf5* gene expression and this was associated with diversion of these cells to the brown fat lineage (Seale *et al.* 2008). In this study, *Myf5* mRNA expression in C2C12 cells was up-regulated in adipogenic media compared with myogenic media at all timepoints over the 6-day culture period, but *Prdm16* mRNA was expressed at very low levels in all cultures, below the threshold at which expression could be accurately quantified (data not shown). Similarly, *Ucp1* expression was not detectable in these cultures (data not shown). Other brown adipocyte-specific marker genes, *Elov13* and *Cidea*, were activated in cells cultured in adipogenic media, but not until day 6 of differentiation, and the expression was a lot more variable, as indicated by the larger error bars. This suggests that activation of brown adipogenic genes may be occurring, but at a much later stage in the developmental process, for which further investigation is required. However, the absence of any effect of 1,25(OH)₂D₃ treatment on any of the brown fat genes measured suggests that the dose-dependent changes in lipid accumulation observed relate to white rather than brown adipogenesis.

**Figure 7**

Dose-dependent effects of $1,25(\text{OH})_2\text{D}_3$ on expression of VDR, CYP27B1 (1α -hydroxylase) and CYP24A1 (24-hydroxylase) enzymes. Expression of VitD-related genes was determined by quantitative RT-PCR analysis. Levels of (A) *Vdr*, (B) *Cyp27b1* and (C) *Cyp24a1* mRNAs were quantified in C2C12 cells cultured in the absence or presence of 10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} or 10^{-5} M $1,25(\text{OH})_2\text{D}_3$ for 2, 4 or 6 days in adipogenic differentiation media. Expression at day 0 (before differentiation media and $1,25(\text{OH})_2\text{D}_3$ was added) is also included for reference and is indicated by a bar. A significant two-way interaction ($P < 0.001$) between day of differentiation and $1,25(\text{OH})_2\text{D}_3$ concentration was observed for VDR only. There was a significant effect of day of differentiation ($P = 0.001$) on expression of *Cyp27b1* mRNA, but no effect of $1,25(\text{OH})_2\text{D}_3$ concentration. By contrast, there was a significant effect ($P < 0.001$) of $1,25(\text{OH})_2\text{D}_3$ concentration on *Cyp24a1* mRNA, but no effect of day of differentiation.

Myogenic differentiation

Our results show that exposure to adipogenic media induces C2C12 cells to transdifferentiate into cells that accumulate lipid droplets and thus resemble a mature adipocyte. This indicates some degree of plasticity in the lineage potential of this 'muscle cell line', but it should be noted that extensive myotube formation was still evident, even in the presence of adipogenic media. Previous studies whereby C2C12 cells were induced to form adipocytes following exposure to thiazolidinediones and fatty acids indicated that this transdifferentiation to mature adipocytes was associated with an inhibition of myogenic differentiation (Teboul *et al.* 1995). An initial myogenesis inhibitory effect of exposing C2C12 cells to adipogenic media was evident in our studies, as CK activity was reduced in control cells exposed to adipogenic compared with myogenic media. This was associated with delayed activation of myogenesis, as indicated by reduced myogenin and *Ck* mRNA at day 2 and was possibly a consequence of increased cell proliferation, as indicated by the increase in DNA content observed, presumably due to the higher FBS content of the adipogenic media. However, this study indicated that the three physiological concentrations (10^{-13} – 10^{-9} M) of $1,25(\text{OH})_2\text{D}_3$ appeared to induce myogenesis (in adipogenic media only), rather than inhibit it. Indeed, the low physiological (10^{-13} M) concentration of $1,25(\text{OH})_2\text{D}_3$ appeared to increase both adipogenesis and myogenesis, whereas the high physiological (10^{-9} M) concentration only increased myogenesis and the highest supraphysiological (10^{-5} M) concentration inhibited both. This is consistent with previous work in which a high physiological concentration (10^{-9} M) of $1,25(\text{OH})_2\text{D}_3$ was shown to increase fusion/differentiation of chick embryo myoblasts during late stages of myogenesis (Capiati *et al.* 1999).

Local regulation of activity

Circulating levels of $1,25(\text{OH})_2\text{D}_3$ are tightly regulated by the activity of the hydroxylating enzymes, 1α -hydroxylase (CYP27b1) and 24-hydroxylase (CYP24a1). In this study, the activating enzyme, *Cyp27b1* mRNA, was found to be expressed by C2C12 cells and expression increased with day/stage of differentiation in adipogenic media, but there was no effect of $1,25(\text{OH})_2\text{D}_3$. Although the highest concentration (10^{-5} M) appeared to reduce expression of *Cyp27b1* mRNA, this was not statistically significant. Previous work (Turunen *et al.* 2007) has shown that $1,25(\text{OH})_2\text{D}_3$ administered at suprananomolar levels

(10^{-8} M) suppressed 1α -hydroxylase (CYP27b1) promoter activity in HEK 293 cells. It should be noted that there was no detectable expression in the C2C12 cells of the inactivating enzyme, CYP24a1 (24-hydroxylase), apart from when $1,25(\text{OH})_2\text{D}_3$ was supplemented at levels in excess of the physiological range (10^{-7} – 10^{-5} M), when an induction was observed. This likely occurs as a mechanism to protect the cells from toxic effects of such high non-physiological levels of the active vitamin. Similarly, VDR mRNA was very low (but detectable) and induced at the highest (10^{-5} M) concentration. This would appear to suggest that there were toxic effects of the highest concentration(s) of $1,25(\text{OH})_2\text{D}_3$, but that this was not the case for the other, more physiological concentrations (10^{-13} – 10^{-9} M), indicating that their effects on morphology and gene expression are unlikely to be via anti-proliferative/pro-apoptotic mechanisms.

Physiological relevance

It is difficult to extrapolate from this study to the likely effects of VitD deficiency on the level of transdifferentiation of muscle precursor cells to adipocytes that occur in muscle *in vivo*. Our findings suggest that low (deficient) levels (Zittermann *et al.* 2009) of $1,25(\text{OH})_2\text{D}_3$ (i.e. 10^{-13} M) may actually enhance adipogenic transdifferentiation, whereas high (sufficient) levels (i.e. 10^{-9} M) inhibit adipogenesis, thereby potentially impacting on fat infiltration and muscle function. A number of studies have demonstrated the ability of primary muscle cells to form adipocytes, but the mechanisms involved are not clear (Asakura *et al.* 2001, Csete *et al.* 2001, Aguiari *et al.* 2008). However, the C2C12 cell model is likely to be a conservative model of adipogenic transdifferentiation, as primary satellite cells isolated from pig muscle demonstrate a much higher degree of plasticity with a greater number of adipocytes and a lower number of myotubes formed in response to adipogenic media (Redshaw *et al.* 2010). The exposure of myogenic precursor cells to adipogenic regulatory factors may be an important factor in contributing to the increased fat infiltration seen in muscle, for example during ageing and VitD deficiency, although infiltration by already committed adipocyte populations is also possible. VitD has been shown to play a key regulatory role in myogenesis and is likely to be important in muscle fibre repair (Capiati *et al.* 1999, Garcia *et al.* 2011).

A speculative interpretation of the bimodal response of C2C12 cells to $1,25(\text{OH})_2\text{D}_3$ observed *in vitro* is that it

represents an energy-conserving mechanism *in vivo* that has evolved in response to the changing seasons and enables extra energy to be repartitioned into fat depots. Certainly for our ancestors, a significant quantity of VitD was primarily obtained from exposure to u.v./sunlight, which induces the conversion of 7-dehydrocholesterol to VitD₃. Low levels of VitD in the body, which possibly occur during periods of low u.v. exposure such as winter (Moosgaard *et al.* 2005, Aguiari *et al.* 2008), may act as an important regulatory cue in inducing muscle precursor cells to form adipocytes rather than myofibres and enable extra fat depots to be stored in the body during periods of austerity. This speculative hypothesis needs testing in an appropriate animal model.

In conclusion, this is the first study to show that low physiological concentrations (0.1–10 pM or 10^{-13} – 10^{-11} M) of $1,25(\text{OH})_2\text{D}_3$, which may represent a VitD-deficient state, induce myoblasts to transdifferentiate into the adipogenic lineage and appears to involve activation of PPAR γ 2. These findings have implications for muscle health and function as well as whole-body energy metabolism because an increase in fat infiltration within skeletal muscle has been linked with a decrease in functional strength and impairment of glucose tolerance, leading to an increased susceptibility to obesity and type II diabetes (Goodpaster *et al.* 2003, Hilton *et al.* 2008). High concentrations (1 nM or 10^{-9} M and above) of $1,25(\text{OH})_2\text{D}_3$ appeared to block adipogenic transdifferentiation, suggesting that changes in physiological concentrations of $1,25(\text{OH})_2\text{D}_3$ have a major impact on the determination of cell fate of myogenic precursor cells. Furthermore, our data indicate that levels of $1,25(\text{OH})_2\text{D}_3$ in the serum and muscle are likely to be important biomarkers linking VitD intakes and optimal muscular health. Given the widespread prevalence of VitD deficiency, particularly in the elderly population, understanding the role of this vitamin in muscle differentiation processes throughout life will be key to defining nutritional parameters for maintaining life-long health and well-being.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-12-0234>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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