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Calcitriol Increases Ceramide, Diacylglycerol, and Expression of Genes Involved in Lipid Packaging in Skeletal Muscle

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CALCITRIOL INCREASES CERAMIDE, DIACYLGLYCEROL AND EXPRESSION OF GENES INVOLVED IN LIPID PACKAGING IN SKELETAL MUSCLE CELLS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Education at the University of Kentucky

By

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Lexington, Kentucky
2016

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ABSTRACT OF THESIS

CALCITRIOL INCREASES CERAMIDE, DIACYLGlycerol AND EXPRESSION OF GENES INVOLVED IN LIPID PACKAGING IN SKELETAL MUSCLE CELLS

Background: Vitamin D is crucial for skeletal muscle function. 25-hydroxyvitamin D (25(OH)D) has been correlated with skeletal muscle mass and intramyocellular lipid (IMCL) content. The purpose of this study was to understand how calcitriol, the active vitamin D metabolite, directly affects myocellular size and lipid partitioning. Methods: C2C12 myotubes were treated with calcitriol (100nM) or vehicle control for 24 or 96 h. Myotube diameter and protein synthesis rate were measured to determine effects of calcitriol on myocellular size. Intramyocellular triacylglycerol (IMTG), diacylglycerol (DAG), and ceramide content were measured by LC/MS. Expression of genes involved in lipid packaging and lipolysis were measured by RT-PCR. Insulin-stimulated phosphorylated Akt (Thr 308) was determined by western blot.

Results: Calcitriol did not affect myocellular size or protein synthesis rate. Calcitriol increased total DAG and ceramides in a sub-species specific manner. Calcitriol increased IMTG area, but did not affect total IMTG content. Calcitriol reduced mRNA content of diglyceride acyltransferase and increased mRNA content of lipid packaging genes. Calcitriol did not negatively affect insulin-stimulated pAkt.

Conclusions: These results suggest calcitriol directly alters lipid content and packaging in skeletal muscle cells. Altering the expression of lipid packaging genes and increasing IMCL subspecies content may be mechanisms by which vitamin D improves skeletal muscle function in vivo.

KEYWORDS: (vitamin D, triacylglycerol, diacylglycerol, lipid packaging, muscle function)

Grace Jefferson

April 13, 2016
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INTRODUCTION

Vitamin D has pleiotropic effects on health including increased muscle strength (25), improved physical function (73), and prevention of muscle atrophy (78). Whole-body vitamin D status, as assessed by serum 25-hydroxyvitamin D3 (25(OH)D), is positively associated with lean body mass (42). Additionally, vitamin D supplementation has been shown to improve physical performance parameters (19, 25, 42, 73). Furthermore, vitamin D appears to be essential for proper skeletal muscle function; vitamin D receptor knock out results in decreased grip strength and altered muscle fibers in mice (45). Furthermore, vitamin D supplementation increases appendicular muscle strength (25), and reduces risk of falls in older individuals (10).

Calcitriol (1,25(OH)₂D₃), the active form of vitamin D, directly regulates transcription of more than 1,000 human genes (26, 91) via the Vitamin D Receptor (VDR). It has recently been determined that the VDR is expressed within skeletal muscle (27, 48, 76), suggesting that vitamin D may directly impact skeletal muscle function. However, the mechanism by which vitamin D improves skeletal muscle function has not been fully elucidated (46). It has been hypothesized that vitamin D may enhance skeletal muscle function by increasing myocellular size (78). Indeed, Girgis et al. (47) recently demonstrated that C2C12 myotubes treated with 100 nM 25(OH)D and 100 nM 1,25(OH)₂D for 7 or 10 days showed increased myotube diameter, suggesting vitamin D may improve muscular strength by inducing myocellular hypertrophy. However, Van der Meijden et al. (62) recently reported that treating C₂C₁₂ myotubes with 100 nM calcitriol did not appreciably increase
myotube diameter or anabolic signaling through the Akt/mTOR pathway, suggesting that vitamin D does not increase myocellular protein synthesis. Therefore, the direct effects of vitamin D on myocellular hypertrophy remain controversial.

It has also been suggested that vitamin D may enhance skeletal muscle function and physical performance by altering intramyocellular lipid (IMCL) content (73). Whole body vitamin D status is correlated with IMCL content in community-dwelling older adults (73). Calcitriol regulates phospholipid metabolism in skeletal muscle from rachitic chicks which has been proposed as a major mechanism by which calcitriol modulates muscle function (12). Ryan et al. (75) showed low doses (10 nM) calcitriol directly increased lipid content of C₂C₁₂ myoblasts. Together, these data suggest vitamin D increases IMCL content.

In addition to increasing IMCL content, vitamin D may enhance muscle function by altering IMCL partitioning between triacylglycerols, diacylglycerols and ceramides. Intramyocellular triglycerides (IMTG) constitute the majority of the lipid droplet within the muscle, and can serve as a readily available substrate for ATP production (4). Additionally, IMTG content is associated with impaired force production (38) and insulin resistance (32, 60) in obese individuals. However, endurance athletes display high amounts of IMTG, yet remain insulin sensitive (36), suggesting that IMTG content alone does not impair muscle function.

IMTG are stored within the highly specialized lipid droplet organelle (4). It has been postulated that the specific proteins covering the lipid droplet (collectively “coatamer” proteins) protect against the development of insulin resistance (15, 67, 81, 96). The perilipin (PLIN) family of proteins associate with the lipid droplet to
promote storage (PLIN 2,3) or lipolysis (PLIN5/Oxidative tissues enriched PAT protein (OXPAT)) of the constitutive lipids (67). Overexpression of PLIN2 promotes increases in IMTG content (15) and improves insulin sensitivity \textit{in vitro} (15). OXPAT overexpression promotes both IMTG accumulation and lipolysis in skeletal muscle (67, 97). PLIN's are thought to alter IMTG storage by enhancing (OXPAT) or repressing (PLIN 2,3) activity of the lipolytic enzymes, adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). The direct effects of vitamin D on IMTG content and PLIN expression remain unclear.

Two other lipid species, diacylglycerols (DAG), and ceramides, have recently been shown to serve as important regulators of skeletal muscle metabolic function (4, 22). DAG are proposed to accumulate during incomplete lipolysis of IMTG (4) and can serve important roles in cell signaling by acting as a second messenger (4). Calcitriol has been shown to stimulate the biphasic formation of DAG in rat skeletal muscle (41). Ceramides have been shown to modify intracellular signaling pathways to slow anabolism (9, 52). Both DAG and ceramide content are closely associated with skeletal muscle and whole-body insulin resistance (3, 4, 22, 59). It appears that DAG and ceramide affect cellular function in a sub-species dependent manner (4, 9, 52). For example, fatty acid chain length appears to be a major determinant of ceramide function (9, 52).

The purpose of this study was to determine the direct effects of calcitriol on myocellular size, IMCL partitioning between TAG, DAG, and ceramides, and lipid droplet packaging. In order to eliminate indirect effects from other, vitamin D-sensitive tissues, we employed a cell culture model (C2C12 myotubes) which has
been used extensively to study skeletal muscle physiology (61, 66, 89). Due to studies showing vitamin D supplementation improves myotube size (47, 62), we hypothesized that calcitriol increases myocellular size and protein synthesis. Additionally, due to data indicating vitamin D status is correlated with IMCL content and that calcitriol increases DAG and ceramide content in chick and rat skeletal muscle cells (13, 41), we hypothesized that calcitriol increases IMCL content, and modifies lipid partitioning and lipid droplet packaging in skeletal muscle.
Literature Review

Skeletal muscle accounts for an estimated 35 - 42% of total body mass (46) and is essential for ambulation, respiration, and activities of daily living. Low levels of skeletal muscle mass and strength are associated with increased risk of disability and death (29, 98). Additionally, lipid content, composition, and fatty acid oxidation within skeletal muscle are highly associated with muscle function. In obese individuals, intramyocellular triglyceride (IMTG) content is correlated with both impaired force production (8) and insulin resistance (60). Recently, diacylglycerol (DAG) and ceramides have been characterized as lipotoxic agents (4) and linked to development of insulin resistance both in vivo (1, 4) and in vitro (79).

Vitamin D therapy has pleiotropic health benefits including increased appendicular muscle strength and reduced risk of falls, mostly in older individuals with a low baseline vitamin D status (10, 69). However, research examining the effects of vitamin D on skeletal muscle function is limited (46–48). Vitamin D status (serum 25(OH)D) is associated with lower incidence of injury (74) and higher levels of lean body mass (42), and muscle mass and strength (51). Furthermore, Girgis et al. (47), have recently shown that 25(OH)D treatment increases myotube diameter, suggesting vitamin D may directly induce myocellular hypertrophy. Therefore, vitamin D appears to enhance skeletal muscle function, potentially by inducing myocellular hypertrophy, which may contribute to improved health outcomes, such as decreased risk of falls (78), with vitamin D therapy.

Vitamin D is fundamental for proper contractile function of skeletal muscle. Curry et al. (34) showed that rabbits, rendered vitamin D deficient by dietary
methods, were substantially weaker than their vitamin D replete counterparts (34). Furthermore, vitamin D repletion restores physical function in vitamin D deficient humans (25, 49). Studies have found that aged human subjects receiving vitamin D therapy had a lower rate of falls (16, 49, 68). Additionally, Glerup et al. (49) demonstrated that, after vitamin D treatment, young Arab women displayed significant improvements in parameters of muscle function, including maximal voluntary contraction, knee and hip extensor muscle power, and single twitch contraction following 3 and 6 months of vitamin D repletion. Therefore, vitamin D therapy appears to improve whole-body and skeletal muscle function, but the cellular mechanisms behind this effect are unclear.

**Vitamin D Metabolism Overview**

Vitamin D₃ (prohormone) is produced in the skin after exposure to sunlight and is hydroxylated in the liver to 25-hydroxyvitamin D₃ (46). In target tissues, 25(OH)D₃ is further hydroxylated to form calcitriol (1,25(OH)₂D₃), the active vitamin D metabolite, via 25-hydroxyvitamin D₃ 1-alpha-hydroxylase (encoded by the Cyp27b1 gene) (47, 76). Traditionally, the major site of calcitriol formation has been thought to be the kidney (46). However, recent data have shown Cyp27b1 mRNA in other cell types, including adipocytes (62, 65) and skeletal muscle cells (62, 85). However, the enzymatic activity of 25-hydroxyvitamin D₃ 1-alpha-hydroxylase in these cells remains unknown. Girgis et al. (47) recently demonstrated that cultured myotubes respond to 25(OH)D treatment, suggesting that these cells have the capacity to convert 25(OH)D to calcitriol.

The cellular mechanism by which vitamin D improves muscle function has
not been fully elucidated. In many tissues, calcitriol binds to the vitamin D receptor (VDR) to exert its biological effects (48, 68, 72), as described by Brumbaugh and Haussler (23). The presence of the VDR has been reported in murine (47, 48), and human (24) muscle cells on the basis of immunohistochemistry (11, 24), equilibrium binding studies (83), and detection of VDR mRNA by RT-PCR (39). Recent evidence shows the VDR is expressed in human skeletal muscle (11, 28, 33). Calcitriol (1,25(OH)$_2$D$_3$) binds to this steroid receptor, which acts as a nuclear transcription factor (70). Vitamin D restriction or VDR ablation significantly decreases muscle fiber cross-sectional area and grip strength in mice (45), suggesting signaling through the VDR is crucial for proper skeletal muscle contractile function. Via the VDR, calcitriol has genomic effects to increase gene transcription which may directly affect muscle function and metabolism (33, 43). Specifically, 100 nmol/L calcitriol has been shown to increase mRNA content of myogenin (62) and several isoforms of myosin heavy chain (62) in cultured myotubes, indicating that calcitriol indeed alters gene transcription in skeletal muscle cells.

Aside from the specific ability of the VDR to translocate from the cytosol to the nucleus in response to calcitriol, conformational changes of the flexible calcitriol molecule also determine the intermediation of genomic and non-genomic actions via its receptor (16, 24). Alvarez et al. (3), summarized that vitamin D receptor gene polymorphisms and vitamin D interactions with the insulin like growth factor system may further influence glucose homeostasis, which could improve muscle function indirectly. Additionally, treatment with calcitriol elicits rapid uptake of
calcium within muscle cells \textit{in vitro} and \textit{in vivo} \cite{7, 35, 43}, which is important for muscle contraction.

\textbf{Lipid Partitioning within Skeletal Muscle Cells}

Whole-body vitamin D status as assessed by circulating serum 25-hydroxyvitamin D$_3$ (25(OH)D), has been associated with intramyocellular lipid (IMCL) content, or the total lipid content within skeletal muscle cells of elderly subjects \cite{73}. Furthermore, calcitriol augments lipid accumulation within C$_2$C$_{12}$ myoblasts \cite{75}, indicating that signaling through the vitamin D pathways may augment IMCL accretion. It has further been reported that vitamin D modifies phospholipids composition within in skeletal muscle which may impact skeletal muscle function \cite{75}.

Intramyocellular triacylglycerol (IMTG), which constitutes the majority of IMCL \cite{4, 56}, are packaged into lipid droplets \cite{17, 96}. IMTG serves as a major substrate for oxidative metabolism, especially during low or moderate-intensity exercise \cite{4}. However, in obese populations, accumulation of IMTG is associated with impaired force production \cite{60} and insulin resistance \cite{30}. Paradoxically, endurance-trained athletes have high levels of IMTG yet maintain contractile function and insulin sensitivity \cite{4, 36}. In fact, Schrauwen et al. \cite{80} showed that IMCL content is increased after only 2 weeks of aerobic training, indicating that accumulation of IMTG is an early adaptation to aerobic training. This could be a beneficial adaptation considering IMTG is known as a substrate for oxidative metabolism during exercise. Therefore, the role of IMTG in muscle dysfunction remains controversial.
The lipid droplet is coated with a variety of proteins (collectively, “coatamer” proteins) that promote either storage (15), or lipolysis (97) of the constitutive IMTG. Therefore, alterations in lipid droplet packaging may have direct effects on how IMTG affects myocellular function. Perhaps the best-described coatamer proteins are the perilipin (PLIN) family. PLIN 2-5 are expressed in skeletal muscle (96) and each isoform appears to uniquely affect lipid metabolism.

PLIN2 and 3 are known to be necessary for lipid storage and expression of these proteins is increased in response to lipid overload (15). Increased PLIN2 expression has been associated with sarcopenia and muscle weakness (31). Furthermore, genetic knockdown of PLIN2 in C2C12 cells has been shown to increase cellular oxidative capacity (15). Interestingly, PLIN2 overexpression has been shown to increase IMTG and ceramide content, and preserve insulin sensitivity in skeletal muscle (15). In fact, PLIN2 (also known as adipophilin) has been proposed to protect against myocellular insulin resistance induced by various fatty acids (95). PLIN2 has been shown to increase co-localization with HSL on muscle lipid droplets with lipolytic stimulation (67, 71), suggesting PLIN2 may enhance lipolysis. In support of this hypothesis, Shepherd et al. (82) found PLIN 2-associated IMTG was preferentially used during one hour of moderate-intensity endurance exercise (82). Furthermore, prolonged endurance-type training increased PLIN2 expression, with increases in IMTG content in diabetic patients (81). This raises the possibility that lipid droplets coated with PLIN2 may be a favorable fuel substrate especially during exercise, which may protect against some of the lipotoxic effects of high lipid exposure/high fat diet.
PLIN5 or the Oxidative tissues enriched PAT protein (OXPAT) is expressed in highly oxidative tissues and during states of increased fatty acid oxidation in skeletal muscle (17). Many studies (67, 96, 97) have reported a correlation between skeletal muscle oxidative capacity and OXPAT content following endurance exercise, suggesting that OXPAT is involved in the regulation of fatty acid oxidation. Additionally, following endurance and sprint interval training, protein content of OXPAT increased in human vastus lateralis muscle (67) indicating that this protein responds to exercise stimuli. Overexpression of OXPAT within skeletal muscle concomitantly increases expression of oxidative genes and IMTG content (15, 97).

Diacylglycerols (DAG) are lipid intermediates that accumulate within the cell during incomplete triacylglycerol hydrolysis. Additionally, DAGs are generated by inositol triphosphate (IP3) cleavage from phosphatidylinositol 4,5-bisphosphate (PIP2) and thus, serve as secondary messengers of cell signaling (4, 93). Excess DAG within skeletal muscle cells has been implicated in development of insulin resistance (58). Endurance training has been shown to decrease DAG levels in human skeletal muscle (22, 36). With this decrease in DAG, insulin sensitivity index was increased in obese human subjects post endurance training (22). Interestingly, highly trained endurance athletes have been shown to have higher levels of DAG compared to sedentary or obese subjects (4), yet remain insulin sensitive. These data suggest a more complex role for DAG in skeletal muscle. Therefore, the role of DAG in skeletal muscle insulin resistance remains controversial. Several recent papers have suggested ceramides, but not DAG, contribute to insulin resistance (1, 79).
Calcitriol has been shown to influence myocellular DAG content. Facchinetti et al. (41) found that in rat skeletal muscle, calcitriol stimulates the biphasic formation of DAG, and that the second phase of DAG formation was inhibited by the absence of extracellular calcium. Calcitriol activated phospholipase D through a mechanism dependent on extracellular calcium and protein kinase C activation, but additional downstream effects of this mechanism, with all of its components remain unclear (41).

Ceramides, a type of bioactive sphingolipid typically found within the cell membrane, act as secondary messengers to inhibit anabolism and/or induce stress stimuli pathways (6, 9, 52). Accumulation of ceramides has been associated with impaired metabolic function (4), specifically by inhibiting Akt (88, 100), a serine/threonine kinase that acts as an intermediate in the insulin signaling pathway (9). Amati et al. (4), found that ceramide content was two-fold higher in muscle from obese, insulin-resistant subjects, compared to normal weight-sedentary and endurance trained subjects. These results are supported by previous studies that reported total ceramide is higher content in muscle of obese subjects (1) and is associated with insulin resistance and a lower oxidative capacity (30, 87).

**Vitamin D Increases Insulin Sensitivity**

Vitamin D supplementation has been shown to improve insulin sensitivity in obese and vitamin D-deficient humans (2, 54, 84). Calcitriol has been shown to increase insulin-stimulated uptake of $[^{3}H]2$-deoxyglucose in C2C12 myotubes following fatty-acid induced insulin resistance (101). Therefore, calcitriol may directly improve skeletal muscle insulin sensitivity.
Conclusions

Vitamin D is necessary for muscle function. Since vitamin D repletion is known to improve muscle force and physical function parameters, it is likely that vitamin D directly increases myocellular size, which is a large determinant of force production. However, research regarding the effects of vitamin D on myocellular size and protein synthesis remains equivocal (45, 47, 62). Additionally, since calcitriol is associated with IMTG content and is known to increase DAG and ceramides, it is possible that calcitriol alters lipid partitioning, packaging, and utilization within skeletal muscle. Calcitriol-induced modulation of myocellular size and/or lipid partitioning and packaging may have significant physiological ramifications for skeletal muscle function.
METHODS

Cell Culture

C₂C₁₂ (ATCC type) myoblasts were cultured in DMEM with 10% FBS, 100IU/ml penicillin/-streptomycin and maintained at 37°C and 5% CO₂. Myoblasts were seeded at a density of 2.5 x 10⁵ cells per well into six-well plates. Once cells were 80% confluent, media was changed to low serum media (DMEM + 2% horse serum) to induce differentiation into myotubes. Following terminal differentiation (~72-96 h), media was supplemented with non-esterified fatty acids (250μM palmitic acid and 250μM oleate) conjugated to BSA (Sigma Aldrich) to mimic circulating lipids in vivo. These fatty acids were chosen because palmitic acid and oleate have been shown to be the most abundant lipid species found in human adipose tissue, circulating triacylglycerols, and circulating non-esterified fatty acids (53) and are usually the two most commonly consumed fatty acids (40).

Additionally, myotubes were treated with calcitriol (100nM) or vehicle control (Ethanol). This dosage of calcitriol has previously been shown to maximally activate VDR in C₂C₁₂ myotubes (43). All experiments were conducted following either 24h or 96h exposure to fatty acids and calcitriol.

Protein Synthesis and Content

Protein synthesis rate was determined by measuring TCA precipitable ³H-tyrosine incorporation as previously described (18). Following 24h of calcitriol treatment, myotubes were incubated with 5 μCi/ml L-[3,5-³H] tyrosine (PerkinElmer Life Sciences) for 2h, washed thoroughly, and proteins precipitated with 10% TCA. Proteins were scraped into microcentrifuge tubes, pelleted, and
solubilized in 0.2N NaOH. Scintillation counting was performed and normalized to total protein content using a standard bicinchoninic acid (BCA) assay (Sigma Aldrich).

**Myotube Diameter and Oil Red O Measurements**

Following 24 and 96h of calcitriol treatment, images of myotubes were collected with an inverted microscope (Zeiss Observer D1) at a 40x magnification. Using Image J software (NCBI), individual myotubes were randomly selected and myocellular diameter was measured. The mean of three measurements per myotube was reported to control for differences in diameter along the myotube axis. Six images were collected for each condition and 5 myotubes from each image were measured (30 myotubes per condition total).

In order to determine the effects of calcitriol on IMTG content, Oil Red O staining was performed following 96 h of calcitriol treatment as previously described (36, 50). Briefly, myotubes were washed with PBS, treated with 4% paraformaldehyde, and incubated in 60% Oil Red O for 10 minutes. After washing residual Oil Red O from myotubes, images were collected using an inverted microscope (Zeiss Observer D1) at 40x magnification. Using Image J software (NCBI), individual myotubes were randomly selected and myocellular area was measured. These same myotubes were converted to threshold images, and the Oil Red O positive area was measured. Oil Red O data were expressed as a percentage of total myotube area to control for differences in myocellular size. Six images were collected from each condition and 5 myotubes from each image were measured (30 myotubes per condition total).
**Liquid chromatography – mass spectroscopy (LC/MS)**

Myocellular diacylglycerol and ceramide subspecies content were measured following 96 h of calcitriol treatment by liquid chromatography-mass spectroscopy (LC/MS) as previously described (44, 57). Samples were delivered to the University of Kentucky Small Molecule Mass Spectrometry Laboratory core facility. The specific DAG subspecies measured were 8:0, 10:0, 14:0, 16:0, 16:0-18:1, 18:0-18:2, 18:0-20:4, 18:1, 18:1-20:0. The specific ceramide subspecies measured were 14:0, 16:0, 18:0, 20:0, 22:0, 24:0, and 24:1. In order to determine whether alterations in ceramide content were due to changes in ceramide synthesis, dihydroceramide (DH-cer) content was also measured. The specific DH-cer subspecies measured were, 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1. Since Oil Red O measures do not directly quantify amount of IMTG, we also analyzed content of 36 individual triacylglycerol sub-species by LC/MS. To control for differences in myocellular seeding density and/or size, LC/MS data were normalized to total phospholipid content as previously described (55).

**Real-time PCR**

Following 24h of calcitriol treatment, total myocellular RNA was isolated by phenol-chloroform extraction and reverse transcribed to cDNA. Quantitative RT-PCR (ABI 7900, SYBRgreen chemistry) was performed using self-designed primers (NCBI algorithms) for genes involved in lipid droplet packaging (PLIN2, PLIN3, and OXPAT) and storage/lipolysis (CGI-58, ATGL, HSL, DGAT). A list of primers used in the present study can be found in Table 1. Primer specificity was confirmed by dissociation curve for all genes tested. Relative gene expression was normalized to
the endogenous control gene Large Ribosomal Protein (RPLPO), which was unchanged by calcitriol treatment. Relative gene expression was calculated using the ΔΔCt method.

**Immunoblots**

In order to determine how calcitriol affects myocellular insulin sensitivity, immunoblots were performed for total Akt (Cell signaling 9272) and phosphorylated Akt (Thr 308) (Cell signaling 5110) following insulin treatment. Myotubes were treated with calcitriol for 96 h and stimulated with 100 μM insulin for 15min. Myotubes were washed twice with PBS and proteins collected in RIPA buffer with protease and phosphatase inhibitors (Sigma Aldrich). Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight in primary antibodies against pAkt and total Akt. Specific bands were detected by enhanced chemiluminescence following treatment with HRP conjugated secondary antibodies. Band density was measured using Image J software.

**Statistical Analysis**

All experiments were conducted with n = 3 and measurements made on at least two independent experiments with the exception of LC/MS. Data are presented as mean ± SE. Statistical comparisons were made using t-test with α = 0.05, using GraphPad Prism 6 (version 6.05). The exception was with myotube diameter and insulin stimulated pAkt where statistical comparisons were analyzed by two-way-ANOVA (Sidak post-test).
RESULTS

100nM Calcitriol increases VDR gene expression.

100nM calcitriol has previously been shown to maximally activate VDR transcription and activity in C2C12 myotubes (43, 85). In order to confirm the effectiveness of this dosage, we treated C2C12 myotubes with 100nM calcitriol for 24h and assessed mRNA content of VDR by RT-PCR. As shown in Figure 1, VDR gene expression was significantly increased 4.66 fold after 24 h treatment with 100nM calcitriol.

Calcitriol does not induce myocellular hypertrophy in vitro.

It has previously been purported that vitamin D improves skeletal muscle mass and strength (25, 68, 72, 86). In order to determine whether calcitriol directly increases myocellular size and protein synthesis, we measured protein synthesis, protein content, and myotube diameter and in C2C12 myotubes treated with calcitriol (100nM) or vehicle control for 24h or 96h. Following 24 h, calcitriol treatment failed to increase myocellular protein synthesis rate (Figure 2A) or protein content (Figure 2B). There was a significant main effect of time to increase myotube diameter. Myotube diameter was significantly greater at 96 h compared to 24 h (p = 0.0005). However, myotube diameter was not significantly increased by calcitriol treatment at either 24 h or 96 h (Figure 2C).

Calcitriol increases ceramide content in a subspecies-specific manner.

Since vitamin D has been associated with IMCL content, we next wanted to determine how calcitriol affects partitioning of lipids between TAG, DAG, and ceramides. In order to accomplish this, we treated C2C12 myotubes with 100 nM
calcitriol for 96 h and measured content of these lipid species by LC/MS. As shown in Figure 3A, calcitriol increased total ceramide content by (1.22 fold, p = 0.019). Content of ceramides containing saturated fatty acids was not significantly altered by calcitriol treatment (Figure 3B). However, content of ceramides containing unsaturated fatty acids was significantly increased (1.30 fold, p = 0.013) following 96 h of calcitriol treatment (Figure 3C). Examination of specific ceramide subspecies revealed the only ceramide subspecies that was significantly altered by calcitriol treatment was Cer-24:1, which was significantly increased (1.30 fold, p = 0.026) (Figure 3D).

Dihydroceramides (DH-cer) function as precursors for ceramide synthesis. In order to assess whether calcitriol alters myocellular ceramide synthesis, we also analyzed content of total DH-cer, as well as individual subspecies of DH-cer by LC/MS. As shown in Figure 3E, total DH-cer content was increased (1.77 fold, p = 0.006) by calcitriol treatment. Interestingly, calcitriol significantly increased DH-cer 24:0 (1.78 fold, p = 0.015) and DH-cer 22:0 (2.52 fold, p = 0.019) (Figure 3F).

**Calcitriol treatment increases myocellular diacylglycerol content.**

In order to determine how calcitriol directly affects myocellular DAG content, C2C12 myotubes were treated with 100nM calcitriol for 96 h and diacylglycerol content was measured by LC/MS. As shown in Figure 4A, calcitriol significantly increased total DAG content (1.87 fold, p = 0.0002). Calcitriol significantly increased content of saturated (2.17 fold, p = 0.0005) (Figure 4B), and mixed DAG, those containing one unsaturated and one saturated fatty acid (1.49 fold, p = 0.0002) (Figure 4C). As shown in Figure 4D, calcitriol significantly increased several...
individual subspecies of DAG including di-14:0 (1.40 fold, p = 0.029), di-16:0 (1.31 fold, p = 0.005), di-18:1 (1.69 fold, p = 0.0006), 16:0-18:1 (1.57 fold, p = 0.001), 18:0-18:2 (1.38 fold, p = 0.0003), 18:0-20:4 (1.73 fold, p = 0.008), and 18:1-20:0 (2.69 fold, p = 0.001). Interestingly, the relative increase in di-18:0 (46.89 fold) in response to calcitriol was substantially greater than any other subspecies measured, suggesting that calcitriol preferentially increases expression of DAG containing di-18:0 (Figure 4E).

**Calcitriol increases IMTG area but does not affect intramyocellular triacylglycerol content.**

We next wanted to assess IMTG content in response to calcitriol treatment. In order to accomplish this, we measured IMTG area (ORO) and TAG content (LC/MS). Representative ORO images can be found in Figure 5A. As shown in Figure 5B, 96 h of calcitriol treatment significantly increased the relative myotube ORO-positive area, indicating an increase in IMTG area. Since ORO does not necessarily quantify IMTG content, we also analyzed content of 36 individual TAG by LC/MS. As shown in Figure 5C, calcitriol did not significantly alter total TAG content.

**Calcitriol increases expression of genes involved in IMTG storage.**

In order to understand how calcitriol affects lipid droplet packaging, C2C12 myotubes were treated with 100 nM calcitriol for 24 h and gene expression of PLIN 2, PLIN 3, OXPAT, CGI-58, ATGL, HSL, and DGAT was analyzed via RT-PCR. After 24 h of calcitriol treatment, DGAT significantly decreased (0.22 fold, p = 0.04), with no significant change in CGI-58 (p = 0.12) or ATGL (p = 0.09) (Figure 6A). Calcitriol
significantly increased both PLIN2 (2.13 fold, p = 0.008) and PLIN3 (1.55 fold, p = 0.007) after 24 h (Figure 6B). Conversely, calcitriol did not significantly alter OXPAT gene expression (1.52 fold, p = 0.16).

**Calcitriol induced changes in pAkt activity.**

In order to determine how calcitriol affects myocellular insulin sensitivity, we performed immunoblots for total and phosphorylated (Thr 308) Akt. Representative immunoblots can be found in Figure 7A. After calcitriol treatment for 96 h and 100μM insulin stimulation for 15 minutes, there was a trend (p = 0.0591) toward increased pAkt in response to insulin, but it failed to reach statistical significance (Figure 7B).
DISCUSSION

The major findings of the present study are: 1) calcitriol directly increases content of DAG and ceramide in a sub-species dependent manner and 2) calcitriol directly alters expression of genes involved in lipid droplet packaging. Together, these data suggest a direct role of vitamin D in regulating lipid packaging and partitioning in skeletal muscle cells.

Vitamin D has previously been associated with IMCL content in elderly adults (73). Furthermore, calcitriol has been found to augment lipid accumulation within of C_{2}C_{12} myoblasts (75). Together, these data suggest that vitamin D may augment IMCL accretion. Historically, higher levels of TAGs, DAGs and ceramides have been associated with insulin resistance (30, 37). However, in the present study, calcitriol did not negatively affect insulin stimulated pAkt.

In our study, we discovered that calcitriol treatment increased expression of the PLIN “coatamer” or lipid packaging proteins, and decreased DGAT, which is directly involved in triglyceride synthesis. Calcitriol did not alter expression of genes involved in triglyceride lipolysis. Shepherd et al. (82), found that PLIN2-containing lipid droplets were preferentially used during one hour of moderate-intensity cycling suggesting that increased PLIN2 expression may increase the ability of these lipid droplets to undergo lipolysis. Therefore, calcitriol-induced increases in PLIN2 and PLIN3 may serve to enhance lipolysis and lipid oxidation.

In the present study calcitriol increased total, saturated and mixed DAG. Myocellular DAG have previously been associated with lipotoxicity and insulin resistance (6, 59). However, Amati et al. (4) demonstrated that, compared to
sedentary individuals, total and mixed DAG content is higher in endurance-trained athletes. Specifically, these athletes displayed exceptionally high levels of di-18:0 DAG. Interestingly, in the present study, calcitriol preferentially increased content of DAG containing di-18:0. Myocellular DAG are often thought to arise from incomplete hydrolysis of IMTG (4). However, it is well established that DAG serve important roles as secondary messengers in cell signaling (4). Morelli et al. (64) showed that calcitriol increases DAG in chick-embryo muscle cells concomitant with increases in inositol trisphosphate, suggesting that rapid increases in DAG may be due to increases phospholipase C activity rather than incomplete IMTG hydrolysis. Accumulation of DAG via this mechanism rather than incomplete IMTG hydrolysis may explain the apparent paradox between concomitant increases in insulin responsiveness and DAG content seen in the present study. Alterations in myocellular lipid composition has been purported to be a major avenue of vitamin D-induced improvements in muscle function (46). It is possible that increases in DAG, especially, those containing di-18:0, may be one mechanism by which calcitriol improves skeletal muscle metabolic function.

In the present study, calcitriol directly increased ceramide content. Ceramides act as secondary messengers in many signaling pathways (6, 9, 52), but their accumulation in muscle has also been associated with impaired insulin resistance (4). In addition to Adams et al. (1) which reported a higher total ceramide content in obese muscle, Amati et al. (4), found that ceramide content was also two-fold higher in insulin-resistant muscle from obese subjects, in comparison to muscle from normal weight-sedentary and endurance trained subjects. A major
mechanism through which ceramide alters cellular metabolism is inhibition of Akt (88, 100), a serine/threonine kinase that acts as an intermediate in the insulin signaling pathway (9). Grosch et al. (52) summarized that ceramides have been shown to have specific chain-length dependent properties, such as short chain dihydroceramides being associated with autophagy (99) and long chain ceramides being associated with apoptosis (92). Interestingly, in the present study, calcitriol only significantly increased content of Cer-24:1. Argraves et al. (5) found an inverse relationship between Cer-24:1 in serum HDL and ischemic heart disease and noted that it may contribute to the protective role of HDL in the disease process (5). It is possible that, unlike other ceramide sub-species, specifically increasing Cer-24:1 may improve muscle metabolic function. Although several papers have measured ceramide sub-species content (4, 5, 20, 44), few, if any, have shown a direct link between individual sub-species and insulin resistance. Future research should be aimed at determining whether alterations in specific lipid sub-species such as di-18:0 DAG and Cer-24:1 mediate the positive effects of vitamin D on skeletal muscle performance in vivo.

It has been previously reported that vitamin D inhibits free fatty acid-induced insulin resistance in skeletal muscle cells (101). IMTG, DAG and ceramide have all been implicated as potential causes of myocellular insulin resistance (59, 94). Therefore, due to the substantial increase in DAG and ceramide in the present study, we wanted to examine how calcitriol affects insulin sensitivity. We found that calcitriol treatment preserved insulin-stimulated Akt phosphorylation at Thr 308 suggesting that calcitriol maintains insulin sensitivity despite increases in
myocellular DAG and ceramide. The specific mechanism(s) underlying calcitriol-induced insulin sensitivity warrant further investigation.

Figure 8 presents a potential mechanism for how calcitriol regulates lipid partitioning/packaging, and insulin resistance. In our model, calcitriol induces translocation of the VDR to the nucleus, which increases transcription of PLIN2 and PLIN3 to increase lipid droplet surface area and increase insulin sensitivity. Since our data show calcitriol increases PLIN2 and PLIN3, but does not alter IMTG content, we postulate that calcitriol increases myocellular lipid droplet number. Previous reports have shown that exercise-induced increases in IMTG content are due to increased lipid droplet number, but not size (90). Since this effect occurs concomitantly with increased fatty acid oxidation, it appears that maximizing lipid droplet surface area may increase myocellular lipolytic potential, limiting any lipotoxic effects of IMTG accumulation. PLIN2 knockdown increases lipid droplet size in an attempt to maximize the volume-to-surface ratio in the available lipid droplets (15). Therefore, it appears PLIN2 may limit lipid size, and maximize surface area of lipid droplets. Maximizing lipid droplet surface area may provide additional opportunities for lipolytic enzymes to mobilize fatty acids from constituent IMTG, thus limiting potential lipotoxic effects of IMTG.

Most (1, 4, 37, 59, 87), but not all (21), studies have shown increased myocellular ceramide content is associated with insulin resistance. Brown et al. (21) demonstrated that increased ceramide levels in the liver did not impair insulin sensitivity, suggesting that ceramide subspecies composition, rather than total ceramide content, may be a more important determinant lipotoxicity. Additionally,
Bosma et al. (15), found that PLIN2 overexpression rescued palmitate-induced insulin resistance, despite increased IMTG and total ceramide content within skeletal muscle. These researchers did not report changes in specific ceramide subspecies. Therefore, the direct effects of PLIN2 on specific ceramide subspecies remain unknown. However, it is possible that PLIN2 increases ceramide content in a subspecies dependent manner.

Previous studies have shown vitamin D status is highly related to physical function (25, 73). Furthermore, calcitriol therapy (77) and supplementation (78) increases skeletal muscle strength, which has been suggested to be due to myocellular hypertrophy (14, 47). Girgis et al. (45) found that VDR knockout and vitamin D-deficient mice had significantly weaker hand grip strength and increased myostatin levels in the quadriceps muscle, which could promote protein degradation and muscle atrophy. Therefore, we wanted to determine how calcitriol affects myocellular size. With our calcitriol treatment (100 nM), we found no change in myotube diameter, protein content, or protein synthesis rate. These data support the findings of Van der Meijden et al. (62) who showed calcitriol had no effect on protein synthetic signaling (pAkt, pP70/pS6) in C2C12 myoblasts. These data indicate that calcitriol does not directly increase myocellular size. However, calcitriol could be mediating its effects indirectly to cause hypertrophy. Garcia et al. (43) demonstrated that the addition of calcitriol to C2C12 myoblasts increased fibroblast growth factor 1 (FGF-1) which promotes vascularization and tissue regeneration, while it decreased fibroblast growth factor 2 (FGF-2), a myogenic inhibitor. It should be mentioned that our findings conflict with those of Girgis et al.
who showed that 10 d of 100 nM calcitriol treatment increased diameter of C2C12 myotubes. Although calcitriol is considered the active vitamin D metabolite, it is possible that the length of calcitriol treatment explains our disparate results (47). Our treatment was terminated at 96h, at which point we observed a small, nonsignificant trend (1.09 fold, p = 0.0578) for calcitriol to increase myotube diameter. It is possible that with a longer duration of calcitriol treatment, we may have observed an increase in myotube hypertrophy. These results suggest a dosage and duration specific effect of calcitriol on myocellular size. To our knowledge, this is the first study to examine the effects of calcitriol on protein synthesis rate in differentiated myotubes.

Vitamin D has long been known to be essential for optimal skeletal muscle function (14, 46, 47, 49, 68). Our results indicate that calcitriol directly alters expression of genes involved in lipid droplet packaging and increases content of DAG and ceramide in a sub-species dependent manner. However, we found no direct effect of calcitriol on myocellular size or protein synthesis in vitro. It is possible that alterations in lipid droplet packaging and lipid subspecies content may be involved in vitamin D-induced improvements in skeletal muscle function. Further research is needed to determine the precise role of calcitriol-induced alterations in lipid partitioning and packaging in skeletal muscle metabolism.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
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| VDR    | F: CCACCACAAGACCTACGACC  
        | R: ATCATGTCAGTGAGGGGT |
| CGI-58 | F: CGGTGGAGGGTCAGGATGG  
        | R: TATGCACACTTTCTTCT  |
| ATGL   | F: GAGGAATGCGCTACTGAACC  
        | R: AGGCTGCAATTGATGCTCCT  |
| HSL    | F: CCTGCCAGTGTTGTAACA  
        | R: AGAAACGCTGAGGCTGTGTACTT  |
| DGAT   | F: AAGAGGACGAGTGCAGACAG  
        | R: GATGGAACCTCAGATCCAGTA  |
| PLIN 2 | F: TCAGCTCTCCTGTAGGAAG  
        | R: TTCTCGGCAATCTCACAC  |
| PLIN 3 | F: ATGAAACACTCCCTCGGCAAG  
        | R: CCTGCTGGGCTCTTCTGTTT  |
| OXPAT  | F: TACTTTGTGCTTGAGGTCT  
        | R: CTTGGTACGGGTGTGTGTCT  |
| RPLPO  | F: ACCCTGAGTCTCGACATC  
        | R: CCGATCTGCAGACACACACT  |

**Table 1. List of real-time polymerase chain reaction (RT-PCR) primers.** VDR = vitamin D receptor. CGI-58 = comparative gene identification – 58. ATGL = adipose triglyceride lipase. HSL = hormone sensitive lipase. DGAT = diglyceride acyltransferase. PLIN 2 = perilipin 2. PLIN 3 = perilipin 3. OXPAT = oxidative tissues enriched PAT protein. RPLPO = large ribosomal protein.
Figure 1. 100 nM calcitriol increases gene expression of the Vitamin D Receptor. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 24 h and VDR gene expression was measured by RT-PCR. Data are expressed as mean ± SE (n=3 per group). *p < 0.05. VDR = Vitamin D Receptor. Veh = vehicle treated. Cal = 100 nM calcitriol.
Figure 2. Calcitriol does not directly induce myocellular hypertrophy. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 24 or 96 h. (A) 24 h of calcitriol treatment did not alter myocellular protein synthesis ($^3$H-tyrosine incorporation). (B) Protein content was unchanged by calcitriol treatment after 24 h. (C) Myotube diameter was unchanged by calcitriol treatment after 24 or 96 h. Data are expressed as mean ± SE (n=3 per group). * p < 0.05 compared to 24 h. Veh = vehicle treated. Cal = 100 nM calcitriol.
Figure 3. Calcitriol increases total ceramide content, specifically ceramide 24:1. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 96 h and ceramide content measured by LC/MS. (A) Total ceramide content was increased by calcitriol treatment after 96 h. (B) Saturated fatty acid ceramide content was unchanged by calcitriol treatment after 96 h. (C) Unsaturated fatty acid ceramide content was increased after calcitriol treatment for 96 h. (D) Ceramide 24:1 was exclusively increased by calcitriol treatment after 96 h. (E) Dihydroceramides, normalized to total phospholipid content, increased with calcitriol treatment after 96 h. (F) Total dihydriceramide content fold change, specifically DH-Cer 24:0, increased after 96 h of calcitriol treatment. Data are expressed as mean ± SE (n=3 per group) and normalized to total phospholipid content. * p < 0.05. Veh = vehicle treated. Cal = 100 nM calcitriol. Cer = ceramide. DHCer = dihydriceramide.
Figure 4. Calcitriol increases total, saturated, and mixed diacylglycerol (DAG) content and certain DAG subspecies. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 96 h and DAG content measured by LC/MS. (A) Total DAG content was increased by calcitriol treatment after 96 h. (B) Saturated DAG content was increased by calcitriol treatment after 96 h. (C) Mixed DAG was increased after calcitriol treatment for 96 h. (D) DAG content of specific subspecies was increased by calcitriol treatment after 96 h. (E) DAG 18:0 increased with calcitriol treatment after 96 h. Data are expressed as mean ± SE (n=3 per group). * p < 0.05. Veh = vehicle treated. Cal = 100 nM calcitriol. DAG=diacylglycerol.
Figure 5. Calcitriol increases intramuscular triacylglycerol area, but not content. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 96 h, and IMTG content assessed by Oil Red O staining and LC/MS. (A) Representative Oil Red O (ORO) staining with vehicle or calcitriol treatment. (B) IMTG area (% covered by ORO) increased with calcitriol treatment after 96 h. (C) Total TAG content was unchanged by 96 h of calcitriol treatment. Data are expressed as mean ± SE of each group (n=3 per group). * p < 0.05. Veh = vehicle treated. Cal = 100 nM calcitriol. IMTG = intramuscular triacylglycerol. TAG = triacylglycerol.
Figure 6. Calcitriol decreases DGAT gene expression and increases PLIN 2 and PLIN3 gene expression. C2C12 myotubes were treated with calcitriol (100 nM) or vehicle control for 96 h and analyzed by real time polymerase chain reaction (RT-PCR) analysis. (A) DGAT gene expression decreased after 96 h calcitriol and lipid treatment. (B) Calcitriol increased PLIN2 and PLIN3 gene expression, while OXPAT was unchanged after 96 h. Data are expressed as mean ± SE of each group (n=3 per group). * p < 0.05 vs. Veh. Veh = vehicle treated. Cal = 100 nM calcitriol. CGI-58 = comparative gene identification-58. ATGL = adipose triglyceride lipase. HSL = hormone sensitive lipase. DGAT = diglyceride acyltransferase. PLIN = perilipin. OXPAT = oxidative tissues enriched PAT protein.
Figure 7. Calcitriol maintains insulin sensitivity during high fat exposure. C2C12 myotubes were treated with a calcitriol (100 nM) or vehicle control with 100μM insulin stimulation for 15 minutes and measured by immunoblots. (A) Representative immunoblot. (B) pAkt normalized to tAkt content increased after calcitriol treatment for 96 h and insulin treatment for 15 minutes, but only in response to calcitriol. Data are expressed as mean ± SE of each group (n=3 per group). * p < 0.05 vs. Basal. Veh = vehicle treated. Cal = 100 nM calcitriol. pAkt = phosphorylated protein kinase B. tAkt = total protein kinase B.
Figure 8. Potential mechanism to explain calcitriol-induced alterations in myocellular lipid content and packaging. Calcitriol increases transcription of lipid packaging genes, specifically PLIN2 and PLIN3, directly by the vitamin D receptor (VDR). PLIN2 and 3 increase lipid droplet surface area (but not IMTG content), ceramide 24:1 content, and preserve insulin sensitivity by increasing lipolytic flux. VDR = vitamin D receptor. PLIN = perilipin.
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