

Leucine and Calcium Regulate Fat Metabolism and Energy Partitioning in Murine Adipocytes and Muscle Cells

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Abstract Dietary calcium modulation of adiposity is mediated, in part, by suppression of calcitriol, while the additional effect of dairy products is mediated by additional components; these include the high concentration of leucine, a key factor in the regulation of muscle protein turnover. We investigated the effect of leucine, calcitriol and calcium on energy metabolism in murine adipocytes and muscle cells and on energy partitioning between adipocytes and skeletal muscle. Leucine induced a marked increase in fatty acid oxidation in C2C12 muscle cells ($P < 0.001$) and decreased FAS expression by 66% ($P < 0.001$) in 3T3-L1 adipocytes. Calcitriol decreased muscle cell fatty acid oxidation by 37% ($P < 0.001$) and increased adipocyte FAS gene expression by threefold ($P < 0.05$); these effects were partially reversed by either leucine or calcium channel antagonism with nifedipine. Co-culture of muscle cells with adipocytes or incubation with 48-h adipocyte conditioned medium decreased muscle fatty acid oxidation by 62% ($P < 0.001$), but treating adipocytes with leucine and/or nifedipine attenuated this effect. Leucine, nifedipine and calcitriol also modulated adiponectin production and thereby exerted additional indirect effects on fatty acid oxidation in C2C12 myotubes. Adiponectin increased IL-15 and IL-6 release by myotubes and partially reversed the inhibitory effects of calcitriol. Comparable effects of leucine, calcitriol and adiponectin were found in myotubes treated with conditioned medium derived

from adipocytes or co-cultured with adipocytes. These data suggest that leucine and nifedipine promote energy partitioning from adipocytes to muscle cells, resulting in decreased energy storage in adipocytes and increasing fatty acid utilization in muscle.

Keywords Calcium · Calcitriol · Leucine · Adiponectin

Introduction

Adipose tissue has previously been perceived predominantly as a fuel reservoir that provides skeletal muscle and other organs with non-esterified fatty acids (NEFA) when exogenous nutrients are insufficient for their energy needs. Accordingly, many previous studies have focused on the development and metabolism of adipose tissue with the final aim of understanding the control of body fat stores. However, adipose tissue is now recognized as an active endocrine organ which synthesizes and secretes a variety of biological molecules, including adiponectin, leptin, tumor necrosis factor alpha (TNF α), interleukins-6 (IL-6) and interleukins-15 (IL-15) [1–4]. The more recent recognition that skeletal muscle may also assume a similar role in response to various metabolic stimuli suggests a potential interaction between skeletal muscle and adipose tissue [5–6].

Previous data from this laboratory demonstrate that dietary calcium exerts an anti-obesity effect by inhibiting calcitriol secretion [7–10]. We have shown that calcitriol mediates increases in intracellular calcium ($[Ca^{2+}]_i$) in adipocytes via the 1, 25(OH) $_2$ -D $_3$ -membrane associated rapid response steroid hormone

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(1, 25D₃-MARRS) binding protein and stimulates lipogenesis and inhibits lipolysis via a calcium dependent mechanism [11–15]. We have also shown a dose responsive inhibition of uncoupling protein 2 (UCP2) expression by calcitriol in human adipocytes mediated by the nuclear vitamin D receptor (nVDR) [16]. Moreover, we have shown that suppression of circulating calcitriol levels by increasing dietary calcium suppresses adipocyte [Ca²⁺]_i, increases UCP2 expression, promotes adipocyte apoptosis in white adipose tissue and reduces metabolic efficiency and adiposity in a mouse model of obesity [7–9], suggesting that dietary strategies designed to suppress circulating calcitriol levels may reduce adiposity.

In support of this concept, dietary calcium suppressed fat gain on eucaloric diets and accelerated fat loss on hypocaloric diets in both animals and humans [7–9]. Notably, providing calcium in the form of dairy, which also provides branch chain amino acids (BCAAs), exerted a greater effect on adiposity and protected muscle mass during energy restriction and increased muscle mass on eucaloric diets [17–18], suggesting that dietary calcium provided with BCAAs may regulate energy partitioning in a tissue selective manner and regulate energy metabolism by modulating endocrine function of both adipose tissue and skeletal muscle, favoring elevated energy expenditure in adipose tissue and promoting protein synthesis in skeletal muscle. However, the effect of BCAAs, specifically leucine, in regulating this process is unclear. Accordingly, the objective of the present study was to determine the role and mechanism of calcium, calcitriol and leucine in regulating cross-talk between adipose tissue and skeletal muscle, and in thereby modulating adiposity.

Material and Methods

Cell Culture

C2C12 and 3T3-L1 preadipocytes (American Type Culture Collection) were incubated at a density of 8,000 cells/cm² (10 cm² dish) and grown in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and antibiotics (adipocyte medium) at 37 °C in 5% CO₂. Confluent 3T3-L1 preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM (1:1, vol/vol) medium supplemented with 1% FBS, 1 μM dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) and antibiotics (1% penicillin–streptomycin). Preadipocytes were maintained in this

differentiation medium for 3 days and subsequently cultured in adipocyte medium. Cultures were re-fed every 2–3 days to allow 90% cells to reach fully differentiation before conducting chemical treatment. For differentiation of C2C12 cells, cells were grown to 100% confluence, changed into differentiation medium (dexamethasone with 2% horse serum and 1% penicillin–streptomycin), and fed with fresh differentiation medium every day until myotubes were fully formed (3 days).

Co-culture of Adipocyte and C2C12

Cells were co-cultured by using transwell inserts with a 0.4 μm porous membrane (Corning) to separate adipocytes and C2C12 muscle cells as described previously [19]. Each cell type was grown independently in the transwell plates. Following cell differentiation and growth to confluence, inserts containing adipocytes were transferred to myotube plates, and inserts containing myotubes were transferred to adipocyte plates. After incubation for 48 hours, the cells in the lower well were harvested for further analysis.

Treatment of Cells

Calcitriol, leucine, nifedipine or/and adiponectin were freshly diluted in medium before treatment. Cells were incubated in serum free medium overnight and then washed with fresh medium, re-fed with medium containing the different treatments (2.5 mM leucine and/or 5 μM nifedipine with or without 10 nM calcitriol or 70 ng/ml adiponectin) and incubated at 37 °C in 5% CO₂ for 48 h before analysis. In some experiments, the supernatants of differentiated 3T3-L1 adipocytes were used to replace the medium of C2C12 myotubes. Cell viability was measured via trypan blue exclusion. At the end of the incubation, culture supernatants were collected and stored at –20 °C until assayed.

Fat Oxidation

Muscle cell fat oxidation was measured as described previously [20], with minor modification. Briefly, C2C12 cell monolayers were rinsed twice with phosphate-buffered saline (PBS) and incubated in substrate mixture containing 22 μM unlabeled palmitate plus 5 μCi [³H]palmitate in Hank's basic salt solution containing 0.5 mg/ml BSA for 2 h. Negative controls were prepared by treating the monolayer with methanol for 30 s to abolish cellular metabolism. The reaction medium was then collected from cell monolayer and treated with 0.2 ml 10% trichloroacetic acid. The

protein precipitate was removed by centrifugation while supernatants were treated with 6 N NaOH and then applied to a poly-prep chromatography column with 1 ml Dowex-1. The $^3\text{H}_2\text{O}$ passed through the column and the following 1 ml of water wash was collected and radioactivity was measured with a liquid scintillation counter.

To determine total cellular protein of cultures used for fatty acid oxidation assays, cell monolayers were harvested after removal of the reaction mixture and subjected to protein measurement using Bradford protein assay reagents.

Total RNA Extraction

A total cellular RNA isolation kit (Ambion, Austin, TX) was used to extract total RNA from cells according to manufacturer's instruction. The concentration and purity of the isolated RNA were measured spectrophotometrically (A_{280}/A_{260} between 1.9 and 2.1) and the integrity of RNA sample were analyzed via BioAnalyzer (Agilent 2100, Agilent Technologies).

Quantitative Real Time PCR

Adipocyte and muscle 18s, fatty acid synthase (FAS), peroxisome proliferator-activated receptor gamma (PPAR gamma) and mitochondrial uncoupling protein 3 (UCP3) were quantitatively measured using an ABI 7300 Real-Time PCR System (Applied Biosystems, Branchburg, NJ) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers and probe sets were obtained from Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression primers and probe set collection according to manufacturer's instruction. Pooled adipocyte total RNA were serial-diluted in the range of 1.5625–25 ng and used to establish a standard curve; total RNAs for unknown samples were also diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were also performed according to the instructions of ABI 7300 Real-Time PCR System and TaqMan Real Time PCR Core Kit. The mRNA quantitation for each sample were further normalized using the corresponding 18s quantitation.

Measurement of Secretion of Cytokines in Culture Supernatants

Concentrations of adiponectin, IL-15 and IL-6 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits were purchased from Assay Designs (Ann Arbor, MI)

and experiments were performed according to the manufacturer's instructions.

Statistical Analysis

All data were expressed as mean \pm SEM. Data from studies were evaluated by one-way or two-way ANOVA, and significantly different group means ($P < 0.05$) were then separated by the least significant difference test using SPSS (SPSS Inc, Chicago, IL).

Results

Both leucine and nifedipine stimulated fatty acid oxidation in C2C12 muscle cells and this effect was attenuated by calcitriol ($P < 0.001$) (Fig. 1a). Adiponectin markedly increased fatty acid oxidation in C2C12 myotubes and restored fatty acid oxidation suppressed by calcitriol in the presence of leucine (Fig. 1a). Comparable effects of leucine, calcitriol and adiponectin were found in myotubes co-cultured with adipocytes (Fig. 1b); however, the presence of adipocytes markedly suppressed fatty acid oxidation. This effect is attributable to secreted factor(s), as a comparable suppression resulted from exposure of the myotubes to adipocyte conditioned medium (Fig. 1c).

Consistent with this, the expression of UCP3 in C2C12 cells was significantly upregulated by leucine, and calcitriol partially inhibited this effect ($P < 0.001$) (Fig. 2). However, nifedipine did not significantly affect UCP3 expression, indicating that leucine and calcitriol regulate UCP3 expression in a Ca^{2+} -independent manner. This is consistent with our previous data which demonstrates that calcitriol regulates uncoupling protein expression via the nVDR and is independent of Ca^{2+} signalling [16].

Leucine and calcitriol exerted complementary regulation on adipocytes. Leucine inhibited FAS ($P < 0.001$) (Fig. 3a) and PPAR gamma ($P < 0.001$) (Fig. 3b) expression in differentiated 3T3-L1 adipocytes while calcitriol stimulated both FAS and PPAR gamma expression and attenuated the effect of leucine on FAS expression and nifedipine exerted the opposite effect.

Our data also demonstrate that leucine, calcium and calcitriol regulate cytokine release from adipocytes and skeletal muscle and suggest that they may play a role in modulating their effect on energy metabolism. Leucine and nifedipine markedly increased adiponectin production in adipocytes while calcitriol exerted the opposite effect (Fig. 4). Adiponectin significantly enhanced IL-15 release in muscle cells compared to

Fig. 1 The effect of leucine, nifedipine, adiponectin and calcitriol on fatty acid oxidation in C2C12 muscle cells. Fatty acid oxidation was determined by palmitate oxidation. C2C12 myotubes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), adiponectin (70 ng/ml) or/and calcitriol (10 nM) for 48 h in basal medium (a), co-cultured with adipocytes (b) or in conditioned medium derived from 48-h incubation with adipocytes (c). Total cell numbers of cultures used for oxidation assays were evaluated by the DNA content. Values are presented as mean \pm SEM, $n = 6$. Means with different letter differ with $P < 0.05$

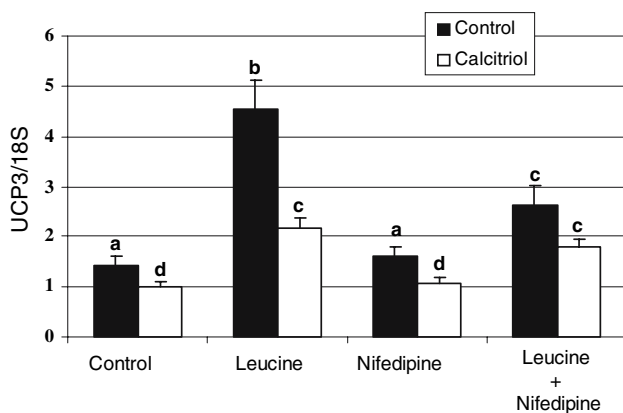
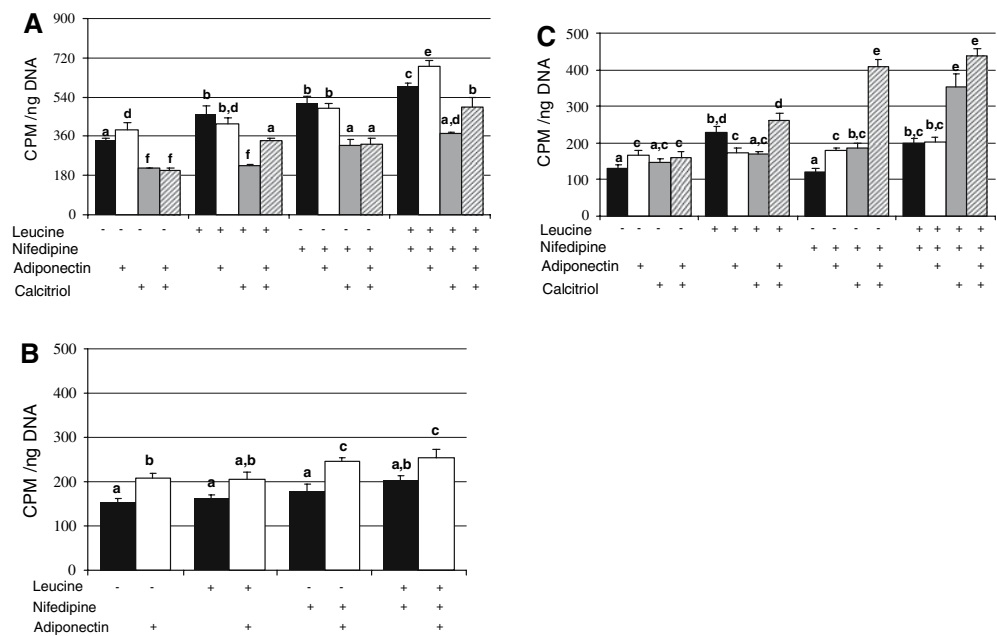


Fig. 2 The effect of leucine, nifedipine and calcitriol on UCP3 to 18s expression ratio in C2C12 muscle cells. C2C12 myotubes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), or/and calcitriol (10 nM) for 48 h. Values are presented as mean \pm SEM, $n = 6$. Means with different letter differ with $P < 0.05$

basal medium and this effect was attenuated by addition of calcitriol (Fig. 5a). Leucine appeared to exert no additional effect on IL-15 release in basal medium with or without adiponectin while nifedipine alone promoted IL-15 release but adiponectin exerted no addition effect. Adiponectin also increased IL-15 release in muscle cells treated with both leucine and nifedipine and this effect was not attenuated by addition of calcitriol, indicating the effect of calcitriol is mediated, at least in part by calcium signaling. Comparable effects were found in myotubes co-cultured with adipocytes (Fig. 5b) or treated with conditioned medium derived from differentiated adipocytes (Fig. 5c).

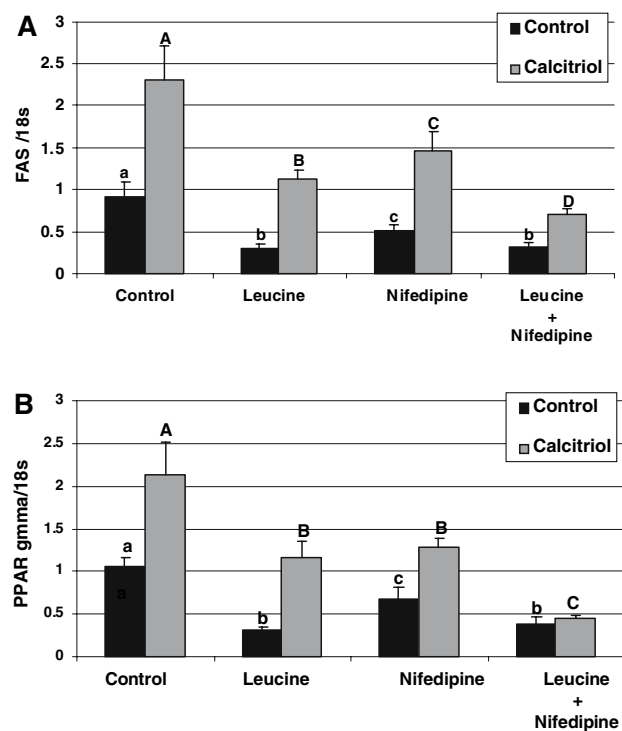


Fig. 3 The effect of leucine, nifedipine and calcitriol on FAS to 18s expression ratio (a) and PPAR gamma to 18s expression ratio (b) in 3T3-L1 adipocytes. Adipocytes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), or/and calcitriol (10 nM) for 48 h. Values are presented as mean \pm SEM, $n = 6$. Means with different letter differ with $P < 0.05$

Similar observations were found in IL-6 production in muscle cells, with nifedipine stimulating IL-6 production, although leucine exhibited no effect (Fig. 6a).

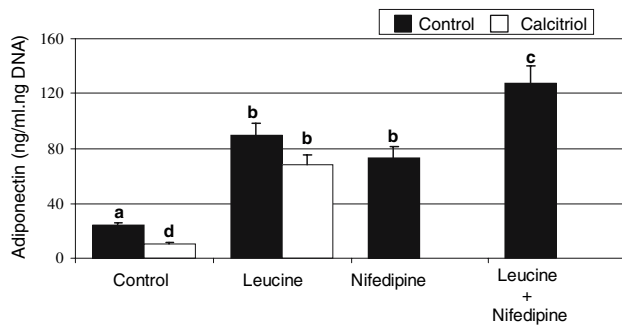


Fig. 4 The effect of leucine, nifedipine and calcitriol on adiponectin production in 3T3-L1 adipocytes. Adiponectin was determined by ELISA as described in “Materials and methods” section. Adipocytes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), or/and calcitriol (10 nM) for 48 h. Total cell numbers of cultures used for adiponectin production were evaluated by the DNA content. Values are presented as mean \pm SEM, $n = 6$. Means with different letter differ with $P < 0.05$

Adiponectin increased IL-6 production and this effect was enhanced by nifedipine but not leucine. Although calcitriol exerted no significant effect on IL-6 production, it attenuated the effect of adiponectin on IL-6 production. Comparable effects were found in muscle cells co-cultured with adipocytes (Fig. 6b) or treated with conditioned medium derived from differentiated adipocytes (Fig. 6c).

Discussion

The present data demonstrate independent roles of leucine and calcium antagonism in regulating energy partitioning between adipocytes and muscle cells, with both favoring fatty acid oxidation and UCP3 expression in C2C12 muscle cells. Calcitriol, a steroid hormone which has been shown to stimulate calcium influx via 1, 25D₃-MARRS, suppressed fatty acid oxidation and attenuated the effects of leucine and nifedipine. Consequently, we hypothesize that this increase in muscle fat oxidation is coupled with decreased fat storage and increased fat catabolism in adipocytes. Consistent with this concept, we found that leucine inhibited FAS and PPAR gamma expression in differentiated 3T3-L1 adipocytes while calcitriol stimulated the expression of both genes and attenuated the effect of leucine on FAS expression, while nifedipine exerted the opposite effect. In addition, muscle cells treated with conditioned medium derived from adipocytes or co-cultured with adipocytes exhibited suppressed fatty acid oxidation, indicating that one or more factors derived from adipocytes regulate skeletal muscle energy metabolism. Indeed, leucine, nifedipine and calcitriol also modulate adiponectin production, with leucine and nifedipine increasing adiponectin production while calcitriol exerted the opposite effect. Consequently, we further evaluated the role of

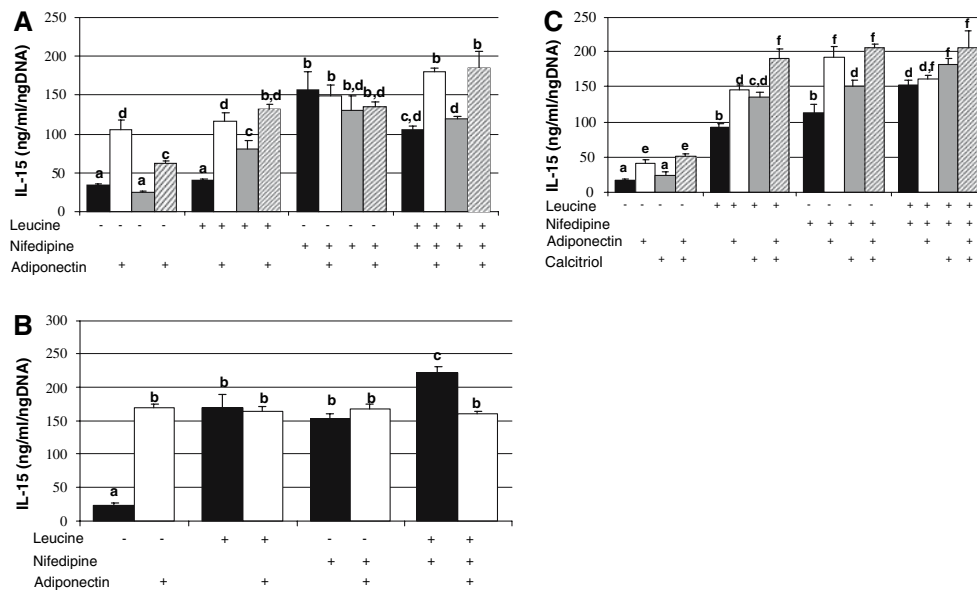


Fig. 5 The effect of leucine, nifedipine, adiponectin and calcitriol on IL-15 production in C2C12 muscle cells. IL-15 production was determined by ELISA and C2C12 myotubes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), adiponectin (70 ng/ml) or/and calcitriol (10 nM) for 48 h in basal

medium (a), co-cultured with adipocytes (b) or in conditioned medium derived from 48-h incubation with adipocytes (c). Total cell numbers of cultures used for oxidation assays were evaluated by the DNA content. Values are presented as mean \pm SEM, $n = 6$. Means with different letter differ with $P < 0.05$

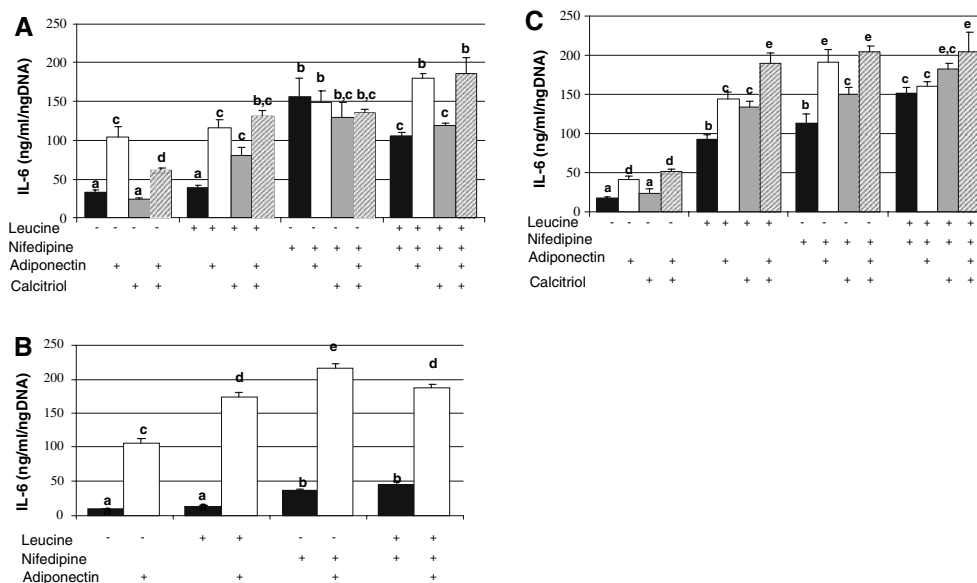


Fig. 6 The effect of leucine, nifedipine, adiponectin and calcitriol on IL-6 production in C2C12 muscle cells. IL-6 production was determined by ELISA and C2C12 myotubes were treated with or without leucine (2.5 mM), nifedipine (10 μ M), adiponectin (70 ng/ml) or/and calcitriol (10 nM) for 48 h in basal

medium (a), co-cultured with adipocytes (b) or in conditioned medium derived from 48-h incubation with adipocytes (c). Total cell numbers of cultures used for oxidation assays were evaluated by the DNA content. Values are presented as mean \pm SEM, $n = 6$. Means with *different letter* differ with $P < 0.05$

adiponectin in mediating the response to leucine, nifedipine and calcitriol. Adiponectin promoted fatty acid oxidation, consistent with previously reported data [21], and enhanced leucine and nifedipine stimulated fatty acid oxidation in muscle cells. This effect was accompanied by up-regulation of IL-15 and IL-6 production in muscle, both of which have been shown to stimulate fatty acid oxidation [22–24].

Adipose tissue excess is an important pathogenic mechanism underlying the obesity-associated metabolic syndrome [25–27]. However, weight loss through energy control is associated with the loss of lean body mass, contributing to decreased energy expenditure [28–29]. This decrease in energy expenditure is of sufficient magnitude to contribute to the very high recidivism following successful weight reduction. Thus, strategies to reduce body fat while also maintaining lean body mass may contribute to successful long-term management of obesity and associated disorders. Previous data demonstrate a role of calcium in regulating adipose tissue lipid metabolism and a role of BCAAs in maintenance of lean mass [18]. However, the effect of calcium and BCAAs in regulation of adipose-muscle cross-talk in modulating energy partitioning had not been evaluated. Data from this study suggest that both leucine and cellular calcium antagonism promote energy partitioning from adipocytes to muscle cells, resulting in decreased energy storage in adipocytes and increasing fatty acid utilization in muscle; moreover,

these effects appear to be partially attributable to modulation of cytokine production.

We have previously demonstrated that high calcium diets inhibit weight and fat gain in both mice and humans on obesigenic diets and accelerate weight and fat loss during energy restriction [17–18]. A key mechanism underlying this anti-obesity effect is suppression of calcitriol, which modulates intracellular Ca^{2+} signaling and mitochondrial uncoupling in adipocytes and consequently results in reduced adiposity and oxidative stress [10–16]. Notably, dairy sources of calcium exert a markedly greater effect on adiposity in both mice and humans [7–9, 30–31]. Moreover, unlike supplementary or fortified sources of calcium, utilization of intact dairy products as a calcium source resulted in a modest increase in muscle mass in the absence of energy restriction and substantially reduced the loss of lean mass which otherwise accompanies hypocaloric diets, while supplementary calcium was without effect on lean mass in either mice or humans. Accordingly, dairy appears to cause a significant change in energy partitioning between adipose tissue and muscle, resulting in reduced energy storage in adipose tissue and increased fat oxidation and energy utilization (presumably for protein synthesis) in skeletal muscle. Provision of BCAAs to skeletal mimics the effect of a complete mixture of amino acids in promoting protein synthesis both in vivo and in vitro [32–33]. For example, incubation of rat diaphragm with BCAAs results in similar

stimulation of protein synthesis to that found with a complete mixture of amino acids [34]. Similarly, perfusion of rat skeletal muscle with BCAAs produces a comparable stimulation of protein synthesis to that found with perfusion of a complete mixture of amino acids [35]. Conversely, perfusion with an amino acid mixture without BCAAs has no effect on protein synthesis. Of the BCAAs, leucine appears to be primarily responsible for this anabolic effect via both mammalian target of rapamycin (mTOR)-dependent and mTOR-independent signaling [36–39]. Leucine inhibits protein degradation in muscle and infusion of leucine in human subjects results in decreased plasma levels of other amino acids [40], indicating that leucine favors protein synthesis by either reducing protein breakdown or increasing amino acid disposal for protein synthesis. Recent data suggest that the effect of leucine in regulation of protein synthesis appears not to be limited to muscle and may regulate protein metabolism in adipose tissue as well [41–42].

Energy partitioning between adipose tissue and skeletal muscle has been previously demonstrated. Animals lacking myostatin exhibit markedly increased skeletal muscle mass and reduced body fat accumulation [43–44]. Comparable results can be achieved by blocking myostatin signaling via c-ski gene overexpression [45]. Moreover, stimulation of beta-adrenergic receptors produces a dramatic increase in skeletal muscle mass and a corresponding reduction body fat content [46–47]. These data suggest an important connection between adipose tissue and skeletal muscle, and *in vitro* evidence from the present study demonstrated that leucine, nifedipine and calcitriol regulate energy metabolism in both adipocytes and muscle cells. These data are consistent with previous *in vivo* observations that dairy increase fat loss but protect lean muscle mass [17]. However, whether leucine and calcium regulate energy partitioning between adipose tissue and skeletal muscle instead of modulating each tissue is yet unclear.

Adipocyte and skeletal muscle derived cytokines may play a key role in the adipocyte-muscle cross-talk in regulating energy partitioning. For example, TNF α , which is expressed and produced by adipocytes and increases in obesity, has been shown to induce muscle wasting via multiple mechanisms [48–50]. On the other hand, leptin and adiponectin, which are also synthesized and secreted by adipose tissue, alter lipid partitioning in skeletal muscle by increasing fat oxidation and decreasing fatty acid incorporation into triacylglycerols [21]. Moreover, adipocytes and muscle both produce IL-6 and *in vitro* studies suggest that IL-6 may stimulate adipocyte fatty acid release and muscle fatty

acid oxidation [22–23], although its *in vivo* effect remains unclear. Notably, IL-15, a cytokine highly expressed in skeletal muscle, decreases fat deposition in adipose tissue but increases skeletal muscle fiber growth [51–52]. Thus, a reciprocal regulation between adipose tissue and skeletal muscle may exist and may control adiposity by regulating the synthesis of fat and protein in adipose tissue and skeletal muscle respectively. Data from this study demonstrate that muscle cells cultured alone exhibit markedly higher fatty acid oxidation than those cultured in conditioned medium derived from adipocytes or co-cultured with adipocytes, suggesting that adipocyte-derived factor(s) participate in the regulation of muscle energy metabolism. In addition, leucine, nifedipine and calcitriol regulate adiponectin production in adipocytes and IL-15 and IL-6 in muscle cells. Administration of adiponectin at a comparable level to that derived from adipocytes stimulated significant increases in fatty acid oxidation and IL-15 and IL-6 production in muscle cells, indicating that adiponectin may play a key role in the metabolic connection between adipose tissue and skeletal muscle. However, the role of additional adipokines is not yet clear.

In conclusion, the present study demonstrates a role for leucine, calcium and calcitriol in the modulation of adipocyte-muscle cross talk in an *in vitro* system. We found leucine and nifedipine to suppress fat anabolism in adipocytes while they promote fatty acid oxidation in muscle cells. Calcitriol, which is increased by low-calcium diets, exerted the opposite effect. These results suggest a potential role of dietary calcium in modulation of energy partitioning between adipose tissue skeletal muscle and that leucine exerts an additional effect on this system.

References

1. Coppack SW (2001) Pro-inflammatory cytokines and adipose tissue. *Proc Nutr Soc* 60:349–356
2. Trayhurn P, Beattie JH (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 60:329–339
3. Ajuwon KM, Jacobi SK, Kuske JL, Spurlock ME (2004) Interleukin-6 and interleukin-15 are selectively regulated by lipopolysaccharide and interferon- γ in primary pig adipocytes. *Am J Physiol Regul Integr Comp Physiol* 286:R547–R553
4. Okamoto Y, Kihara S, Funahashi T, Matsuzawa Y, Libby P (2006) Adiponectin: a key adipocytokine in metabolic syndrome. *Clin Sci (Lond)* 110:267–278
5. Chan MH, Carey AL, Watt MJ, Febbraio MA (2004) Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability. *Am J Physiol Regul Integr Comp Physiol* 287:R322–R327

6. Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK (2002) IL-6 and TNF- α expression in, and release from, contracting human skeletal muscle. *Am J Physiol Endocrinol Metab* 283:E1272–E1278
7. Zemel MB (2005) Calcium and dairy modulation of obesity risk. *Obes Res* 13:192–193
8. Zemel MB, Richards J, Milstead A, Campbell P (2005) Effects of calcium and dairy on body composition and weight loss in African-American adults. *Obes Res* 13:1218–1225
9. Zemel MB, Shi H, Greer B, DiRienzo D, Zemel PC (2000) Regulation of adiposity by dietary calcium. *FASEB J* 14:1132–1138
10. Sun X, Zemel MB (2006) Dietary calcium regulates ROS production in ap2- agouti transgenic mice on high-fat/high-sucrose diets. *Int J Obes (Lond)* 30:1341–1346
11. Shi H, Halvorsen YD, Ellis PN, Wilkison WO, Zemel MB (2000) Role of intracellular calcium in human adipocyte differentiation. *Physiol Genomics* 3:75–82
12. Xue B, Greenberg AG, Kraemer FB, Zemel MB (2001) Mechanism of intracellular calcium ([Ca²⁺]_i) inhibition of lipolysis in human adipocytes. *FASEB J* 15:2527–2529
13. Shi H, Norman AW, Okamura WH, Sen A, Zemel MB (2001) 1 α ,25-dihydroxyvitamin D₃ modulates human adipocyte metabolism via nongenomic action. *FASEB J* 15:2751–2753
14. Xue B, Moustaid N, Wilkison WO, Zemel MB (1998) The agouti gene product inhibits lipolysis in human adipocytes via a Ca²⁺-dependent mechanism. *FASEB J* 12:1391–1396
15. Kim JH, Mynatt RL, Moore JW, Woychik RP, Moustaid N, Zemel MB (1996) The effects of calcium channel blockade on agouti-induced obesity. *FASEB J* 10:1646–1652
16. Shi H, Norman AW, Okamura WH, Sen A, Zemel MB (2002) 1 α ,25- dihydroxyvitamin D₃ inhibits uncoupling protein 2 expression in human adipocytes. *FASEB J* 16:1808–1810
17. Zemel MB (2005) The role of dairy foods in weight management. *J Am Coll Nutr* 24:537S–546S
18. Zemel MB, Miller SL (2004) Dietary calcium and dairy modulation of adiposity and obesity risk. *Nutr Rev* 62:125–131
19. Suganami T, Nishida J, Ogawa Y (2005) A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 25:2062–2068
20. Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I (2005) Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. *Am J Physiol Regul Integr Comp Physiol* 288:R708–R715
21. Dyck DJ, Heigenhauser GJ, Bruce CR (2006) The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. *Acta Physiol (Oxf)* 186:5–16
22. Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA, Hauner H (2001) Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. *J Clin Endocrinol Metab* 86:2281–2288
23. Bruce CR, Dyck DJ (2004) Cytokine regulation of skeletal muscle fatty acid metabolism: effect of interleukin-6 and tumor necrosis factor- α . *Am J Physiol Endocrinol Metab* 287:E616–E621
24. Almendro V, Busquets S, Ametller E, Carbo N, Figueras M, Fuster G, Argiles JM, Lopez-Soriano FJ (2006) Effects of interleukin-15 on lipid oxidation: disposal of an oral [(14)C]-triolein load. *Biochim Biophys Acta* 1761:37–42
25. McPherson R, Jones PH (2003) The metabolic syndrome and type 2 diabetes: role of the adipocyte. *Curr Opin Lipidol* 14:549–553
26. Vega GL (2004) Obesity and the metabolic syndrome. *Minerva Endocrinol* 29:47–54
27. Weiss R, Dziura J, Burgert TS, Tamborlane WV, Taksali SE, Yockel CW, Allen K, Lopes M, Savoye M, Morrison J, Sherwin RS, Caprio S (2004) Obesity and the metabolic syndrome in children and adolescents. *N Engl J Med* 350:2362–2374
28. Ma SW, Foster DO (1986) Starvation-induced changes in metabolic rate, blood flow, and regional energy expenditure in rats. *Can J Physiol Pharmacol* 64:1252–1258
29. Milan G, Dalla, Nora. E., Pilon C, Pagano C, Granzotto M, Manco M, Mingrone G, Vettor R (2004) Changes in muscle myostatin expression in obese subjects after weight loss. *J Clin Endocrinol Metab* 89:2724–2727
30. Causey KR, Zemel MB (2003) Dairy augmentation of the anti-obesity effect of calcium in ap2-agouti transgenic mice. *FASEB J* 17:A746 (abstract)
31. Sun X, Zemel MB (2004) Calcium and dairy products inhibit weight and fat regain during ad libitum consumption following energy restriction in Ap2-agouti transgenic mice. *J Nutr* 134:3054–3060
32. Rennie MJ, Bohe J, Smith K, Wackerhage H, Greenhaff P (2006) Branched-chain amino acids as fuels and anabolic signals in human muscle. *J Nutr* 136:264S–268S
33. Kobayashi H, Kato H, Hirabayashi Y, Murakami H, Suzuki H (2006) Modulations of muscle protein metabolism by branched-chain amino acids in normal and muscle-atrophying rats. *J Nutr* 136:234S–236S
34. Fulks RM, Li JB, Goldberg AL (1975) Effects of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J Biol Chem* 250:290–298
35. Li JB, Jefferson LS (1978) Influence of amino acid availability on protein turnover in perfused skeletal muscle. *Biochim Biophys Acta* 544:351–359
36. Garlick PJ (2005) The role of leucine in the regulation of protein metabolism. *J Nutr* 135:1553S–1556S
37. Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS (2000) Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr* 130:139–145
38. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR (2000) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* 130:2413–2419
39. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC (2002) Tissue- specific effects of chronic dietary leucine and nor-leucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab* 283:E824–E835
40. Nair KS, Short KR (2005) Hormonal and signaling role of branched-chain amino acids. *J Nutr* 135:1547S–1552S
41. Roh C, Han J, Tzatsos A, Kandror KV (2003) Nutrient-sensing mTOR- mediated pathway regulates leptin production in isolated rat adipocytes. *Am J Physiol Endocrinol Metab* 284:E322–E330
42. Lynch CJ, Patson BJ, Anthony J, Vaval A, Jefferson LS, Vary TC (2002) Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue. *Am J Physiol Endocrinol Metab* 283:E503–E513
43. Tobin JF, Celeste AJ (2005) Myostatin, a negative regulator of muscle mass: implications for muscle degenerative diseases. *Curr Opin Pharmacol* 5:328–332
44. McPherron AC, Lee SJ (2002) Suppression of body fat accumulation in myostatin-deficient mice. *J Clin Invest* 109:595–601
45. Suttrave P, Kelly AM, Hughes SH (1990) ski can cause selective growth of skeletal muscle in transgenic mice. *Genes Dev* 4:1462–1472

46. Yang YT, McElligott MA (1989) Multiple actions of beta-adrenergic agonists on skeletal muscle and adipose tissue. *Biochem J* 261:1–10
47. Spurlock ME, Cusumano JC, Ji SQ, Anderson DB, Smith CK 2nd, Hancock DL, Mills SE (1994) The effect of raclopramide on beta-adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J Anim Sci* 72:75–80
48. Coletti D, Moresi V, Adamo S, Molinaro M, Sassoon D (2005) Tumor necrosis factor-alpha gene transfer induces cachexia and inhibits muscle regeneration. *Genesis* 43:120–128
49. Meadows KA, Holly JM, Stewart CE (2000) Tumor necrosis factor-alpha-induced apoptosis is associated with suppression of insulin-like growth factor binding protein-5 secretion in differentiating murine skeletal myoblasts. *J Cell Physiol* 183:330–337
50. Fong Y, Moldawer LL, Marano M, Wei H, Barber A, Manogue K, Tracey KJ, Kuo G, Fischman DA, Cerami A (1989) Cachectin/TNF or IL-1 alpha induces cachexia with redistribution of body proteins. *Am J Physiol* 256:R659–R665
51. Busquets S, Figueras MT, Meijssing S, Carbo N, Quinn LS, Almendro V, Argiles JM, Lopez-Soriano FJ (2005) Interleukin-15 decreases proteolysis in skeletal muscle: a direct effect. *Int J Mol Med* 16:471–476
52. Carbo N, Lopez-Soriano J, Costelli P, Alvarez B, Busquets S, Baccino FM, Quinn LS, Lopez-Soriano FJ, Argiles JM (2001) Interleukin-15 mediates reciprocal regulation of adipose and muscle mass: a potential role in body weight control. *Biochim Biophys Acta* 1526:17–24