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Body fat loss induced by calcium in co-supplementation with conjugated linoleic acid is associated with increased expression of bone formation genes in adult mice☆,☆☆,★

Alice Chaplin, Andreu Palou*, Francisca Serra

Laboratory of Molecular Biology, Nutrition and Biotechnology, University of the Balearic Islands and Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Palma de Mallorca, Spain

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Abstract

The potential of conjugated linoleic acids (CLA) and calcium in weight management in animal models and human studies has been outlined, as well as their use to prevent bone loss at critical stages. In addition, it has been suggested that bone remodeling and energy metabolism are regulated by shared pathways and involve common hormones such as leptin. We have previously shown that supplementation with CLA and calcium in adult obese mice decreases body weight and body fat. The aim of the present study was to assess the effects of these two compounds on bone and energy metabolism markers on bone. Mice (C57BL/6J) were divided into five groups according to diet and treatment (up to 56 days): control (C), high-fat diet (HF), HF+CLA (CLA), HF+calcium (Ca) and HF with both compounds (CLA+Ca). At the end of treatment, bone formation markers were determined in plasma and expression of selected bone and energy markers was determined in tibia by quantitative polymerase chain reaction. Results show that CLA was associated with decreased tibia weight and minor impact on bone markers, whereas calcium, either alone or co-supplemented with CLA, maintained bone weight and promoted the expression of bone formation genes such as bone gamma-carboxyglutamate protein 2 (Bglap2) and collagen Iα1 (Col1a1). Furthermore, it had a significant effect on key players in energy metabolism, in particular leptin and adiponectin tibia receptors. Overall, in addition to the weight loss promoting properties of calcium, on its own or co-supplemented with CLA, our results support beneficial effects on bone metabolism in mice.

Keywords: Bone; Calcium; CLA; Obesity; Osteocalcin

1. Introduction

In recent years, it has been suggested that the skeleton should be considered an endocrine organ due to its close relationship with energy metabolism, as well as with glucose and fat management. A decade ago, it was proposed that both bone remodeling and energy metabolism are regulated by common hormones and further studies have proved that leptin, which appeared in evolution together with the skeleton, is involved in the regulation of bone (see review [1]). In addition, obesity can offer some protection against the development of osteoporosis, although adverse effects of mechanical loading caused by excess body weight are also apparent [2,3].

The main biochemical marker of bone formation processes is osteocalcin (OC), an osteoblast-specific, noncollagenous, vitamin K-dependent molecule. Woo et al. [4] showed that bone regulates energy metabolism using an OC knockout mice model, in which animals were glucose intolerant and overweight. Further studies have shown that this animal model also shows lower pancreatic β-cell proliferation, decreased insulin production and hyperglycemia, effects that are reversed when given OC [5–7]. On the other hand, human studies have shown that patients with obesity and/or metabolic syndrome presented lower concentrations of OC in serum, as well as showing a negative association between OC and body weight, body mass index, fasting triglycerides, plasma glucose, insulin and HOMA (Homeostasis Model Assessment) [8–11]. Furthermore, the metabolic activity of OC is in turn regulated by leptin [12]. Together, this evidence supports the role of skeleton as an endocrine organ contributing to the regulation of energy balance.

Studies have shown that feeding a high-fat (HF) diet can result in impaired bone formation [2,13]. On the other hand, it has been seen that both calcium and conjugated linoleic acids (CLAs) may have a positive effect on bone metabolism in cell cultures [14,15] and mice [12,16–18], particularly on their potential in the prevention of postmenopausal bone loss and osteoporosis. This is of interest when considering their well-documented role on energy metabolism by

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* Corresponding author at: Laboratorio de Biología Molecular, Nutrición y Biotecnología (Nutrigenómica), Universitat de les Illes Balears, Palma de Mallorca, Spain. Tel.: +34 971173170; fax: +34 971173426.

E-mail address: andreu.palou@uib.es (A. Palou).

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promoting weight loss and reducing adiposity levels in a dose-dependent manner [19–23]. Therefore, the aim of this study has been to assess whether calcium and CLA had an added effect on bone and energy-related metabolic markers in tibia in obese animals. To our knowledge, this is the first *in vivo* study that looks into the effects of a dietary co-supplementation with CLA and calcium regarding energy-associated gene expression in bone.

2. Materials and methods

2.1. Animals, diets and supplementation

Male mice (C57BL/6J) were obtained from Charles River (Barcelona, Spain) at 5 weeks of age, weighing 21±0.1 g. Animals underwent an acclimatization period of 1 week and were then divided into groups of four ensuring equal weight average. Housing conditions followed standard procedure and were kept under a 12-h light/dark cycle at 22°C with food and water *ad libitum*. Research Diets Inc (New Brunswick, NJ, USA) provided all diets used and these were presented as pellets to the animals. Detailed composition can be found in Ref. [23]. In brief, diets contained equal proportion of protein (20% kJ) and carbohydrate was used to adjust the energy content. The standard normal-fat diet (NF; 12% kJ as fat) was used as control and an HF diet (43% kJ as fat) was used to induce obesity; both diets contained 4 g/kg of calcium. An HF diet enriched with calcium (12 g/kg) (Ca group) was used to prevent fat gain [21,23].

Mice were divided into five groups (n=8) and were administered the following diets and treatments for 54 days: a NF diet, a HF diet and a Ca diet. In addition, a daily oral dose of CLA (6 mg, equivalent to 21.4 nmol/isomer/day) was given to HF animals (CLA group) and to those following the calcium-enriched diet (CLA+Ca group). Tonalin TG 80 (kindly provided by Cognis) is derived from safflower oil and was used as the CLA supplement. It is composed of triglycerides containing approximately 80% CLA, with a 50:50 ratio of the active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12.

Food was changed twice a week and intake and body weight were registered every 3 days. Body weight, body composition and metabolic parameters (plasma leptin, insulin, glucose and HOMA) have been previously published [23].

All procedures carried out were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (approval 13th February 2006), and university guidelines for the use and care of laboratory animals were followed.

2.2. Sacrifice and sample collection

Sacrifice of mice was carried out within the animal facilities, at the beginning of the light cycle and after 10 h of starvation. An intraperitoneal injection made up of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) was used as anesthetic. Afterward, blood samples were collected by cardiac puncture using heparinized syringes and needles (0.2% heparin diluted with saline; Sigma, Madrid, Spain). To obtain the plasma, collected blood was centrifuged at 1000×*g* for 10 min and stored at −20°C until analysis. White adipose tissue (WAT) (including epididymal, retroperitoneal, mesenteric and inguinal adipose tissue) and tibia of animals were excised, weighed and cleaned with DEPC 0.1%. Bones were divided into two parts using diagonal pliers and snap frozen at −80°C.

2.3. Determination of plasma parameters

Determination of plasma proteins using enzyme-linked immunosorbent assays was carried out using commercial kits: undercarboxylated OC [uOC; Mouse Undercarboxylated Osteocalcin ELISA Kit (EMELCA Bioscience, the Netherlands)], osteocalcin [OC; Osteocalcin ELISA for mice (Uscn Life Science Inc., USA)] and propeptide of 1 collagen C-propeptide [P1CP; RatLaps ELISA (IDS Ltd, UK)].

2.4. Tibia RNA extraction, retrotranscription and real-time polymerase chain reaction

RNA extraction protocol was based on a previously published protocol [24]. Approximately 200 mg of tibia was ground by mortar and pestle, and homogenized with Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) at full speed for 45 s. Samples were then centrifuged at 8600×*g* for 15 s at room temperature. RNA was extracted from the supernatant with the RNeasy Mini Kit (Qiagen, UK). Isolated RNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Samples were retrotranscribed and real-time polymerase chain reaction (PCR) was carried out for the analysis of gene expression in tibia. Briefly, 0.25 μg of total RNA was denatured at 65°C for 10 min and then reverse-transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min and 5 min at 95°C in a thermal cycler (Applied Biosystems 2720 Thermal Cycler, Madrid, Spain). PCR was performed with diluted cDNA template, forward and reverse primers (ranging from 2.5 to 10 μM) and Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). All primers were designed and obtained from Sigma Aldrich Química SA (Madrid, Spain), except bone gamma-carboxyglutamate protein 2 (Bglap2) [25], insulin receptor (Insr) [26] and osteotesticular protein tyrosine phosphatase (OST-PTP) (G. Karsenty, personal communication, 2012). Table 1 includes accession numbers and primer sequences. Real-time PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems) with the following template: 10 min at 95°C followed by 42 temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products amplified, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0). Glutamate oxaloacetate transaminase 2, mitochondria (Got2) and tripartite motif-containing 27 (Trim27) were used for normalization as reference genes. The relative expression of each gene analyzed is presented as percentage of the expression found in NF mice using the $2^{-\Delta\Delta Ct}$ method [27].

2.5. Statistical analysis

Data are presented as means±S.E.M. Equality of variances between groups was assessed by Levene's test. When homogeneity of variances was assumed, one-way analysis of variance (ANOVA) was used to determine the significance of the different parameters between groups. If there was a significant difference, a Bonferroni test was used to determine where the difference lay and to correct for multiple testing. When homogeneity of variances was not assumed, data were log transformed. Linear relationships between key variables were tested using Pearson's correlation coefficients. Threshold of significance was set at $P<.05$. The analysis was performed using the SPSS program for Windows version 21.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Calcium co-supplementation with CLA counteracts obesity induced by HF feeding preventing bone loss

Significant differences were observed among groups regarding WAT weight (P=.000). HF diet was associated with increased adipose tissue in comparison to the rest of the groups, which was not totally counteracted by CLA supplementation (P=.000 vs. NF), but calcium-enriched diet and in co-supplementation with CLA (Ca and CLA+Ca groups) were able to maintain control levels (Fig. 1A). Impact on bone weight was observed in the groups studied (P=.012). CLA-supplemented animals presented lower tibia weight (19% less, P=.015 vs. NF), whereas calcium prevented tibia weight loss (Fig. 1B).

3.2. Plasma OC levels are decreased by co-supplementation of calcium with CLA and correlate positively with WAT weight

Relevant plasma bone biomarkers were analyzed. Significant differences were found among groups for OC (P=.010), which was particularly lower in animals co-supplemented with CLA and calcium (P=.041 vs. NF). Furthermore, OC in plasma was positively correlated with WAT (r=0.377, P=.021) (Fig. 2A). However, high variability was observed between groups and no differences were found concerning uOC and P1CP concentrations in plasma (Fig. 2B).

3.3. Both CLA and calcium supplementation stimulate OST-PTP expression, whereas in co-supplementation, they induce an increase in the expression of bone formation markers

Gene expression of selected bone metabolism genes was determined in mouse tibia. OST-PTP, Bglap2 and Col1a1 messenger RNA (mRNA) expression showed significant changes throughout groups (P=.000, P=.016 and P=.005, respectively), whereas no effects were observed for Fgf23. OST-PTP expression was higher in animals which received either CLA (2.4-fold, P=.000) or calcium (1.8-fold, P=.024) compared to NF, and according to its proposed role as a regulator of bone growth, mRNA levels were negatively correlated with tibia weight (r=−0.410, P=.016). Furthermore, positive correlations were found between expression of OST-PTP and plasma glucose (r=0.542, P=.001) and HOMA index (r=0.436, P=.009) (Fig. 3A). On the other hand, Bglap2 mRNA was significantly increased in animals receiving calcium supplementation, alone or together with CLA (P=.026 and P=.035 vs. NF, respectively). This profile, which could suggest higher bone formation potential, was accompanied by an

Table 1
Nucleotide sequences of primers used for PCR amplification in tibia[a]

	Gene symbol	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)	Accession no.
Housekeeping genes	*Got2*	tctgcctctgccaatcgtatgcc	gagaagtcaggatggttgctgcg	NM_010325.2
	Trim27	gcggagactaacgtgtcgtg	gggcatctggtcctgctc	NM_009054.3
Bone metabolism	*Bglap2*	ctgacctcacagatcccaagc	tggtctgatagctcgtcacaag	NM_001032298.2
	OST-PTP	cagtcactccagcaaggtca	caatgcgctgttcagacagt	AF300701.1
	Fgf23	atgctagggacctgccttaga	agccaagcaatggggaagtg	NM_022657.4
	Col1a1	gctcctcttaggggccact	ccacgtctcaccattgggg	NM_007742.3
Energy metabolism	*Lep*	ttgtcaccaggatcaatgaca	gacaaactcagaatggggtgaag	NM_008493.3
	Lepr	cctgtgcacattcccagccca	actggaacggaaccttgaggcttt	NM_146146.2
	Adrb2	actcaggaacgggacgaag	gcacacgccaaggagattat	NM_007420.3
	Ins1	accgtgtaaatgccactgaa	cggatggactgtttgtaacct	NM_008386.3
	Insr	ccaaccatctgtaagtcaca	acatcaagttgctggaatcatg	NM_010568.2
	Adipor1	aggaagaggaggaggaggtg	gaaaggagggcataggtggt	NM_028320.3
	Adipor2	tacacacagagacgggcaac	aaaaggaaaggcagagaatgg	NM_197985.3

[a] Adipor1: adiponectin receptor 1; Adipor2: adiponectin receptor 2; Adrb2: adrenergic receptor, beta 2; Bglap2: bone gamma-carboxyglutamate protein 2; Col1a1: collagen, type I, alpha 1; Fgf23: fibroblast growth factor 23; Got2: glutamate oxaloacetate transaminase 2, mitochondria; Ins1: insulin; Insr: Insulin receptor; Lep: leptin; Lepr: leptin receptor; OST-PTP: osteotesticular protein tyrosine phosphatase; Trim27: tripartite motif-containing 27.

increase in Col1a1 mRNA levels, particularly in CLA+Ca animals (P=.026 vs. NF) (Fig. 3B).

3.4. Both CLA and calcium supplementation have a significant impact on energy metabolism-associated genes' expression in tibia

The effect of calcium and CLA supplementation on the expression of energy-related genes was determined in tibia. Dietary intervention had a significant impact on the expression of the leptin and insulin receptors [Lepr (P=.000) and Insr (P=.004), respectively], whereas no effect was observed on the expression of their respective targets. HF diet caused a decrease in expression of Lepr (P=.034 vs. NF), which was not recovered in either CLA (P=.025 vs. NF) or CLA+Ca (P=.034 vs. NF) groups; however, calcium supplementation was able to restore control levels. Interestingly, Lepr expression in tibia showed a significant negative correlation with leptin levels in plasma (r=−0.326, P=.049) (Fig. 4A). Concerning adiponectin receptor mRNA levels, dietary intervention modulated the expression of the Adipor1 (P=.011), particularly in calcium-supplemented animals (P=.009 vs. NF), whereas the rest of the groups maintained control values (Fig. 4D). No effects were observed concerning expression of Adrb2 (Fig. 4B) and Adipor2 (Fig. 4B).

4. Discussion

The idea that the skeleton acts as an endocrine organ has been recently put forward, suggesting that it plays a key role integrating whole-organism homeostasis in its interaction with other tissues and systems, being regulation of energy metabolism one of the novel aspects under extensive study [1]. A number of studies have underlined the potential of CLA and calcium in weight management in both animals [19–23] and humans [28,29]. In addition, their use to prevent bone loss at critical stages has raised some interest in the past few years [12,16–18]. Our data show that under dietary conditions that induce obesity such as the HF diet, both oral supplementation of CLA and an enriched calcium diet have a significant effect on expression of key genes associated with both bone and energy metabolism. However, the most interesting results were obtained when CLA was given in co-supplementation with calcium. In this regard, CLA animals exhibited decreased tibia weight, but when given together with calcium, bone weight was maintained, expression of bone formation markers was stimulated and this was associated with counteraction of body fat accretion and body weight gain. Because these compounds are usually used independently to aid in weight loss strategies, these results are suggestive that a joint combination would show higher benefits.

Concerning the molecular mechanisms involved, levels of OC were assessed, being the hormone that contributes to the cross-talk between bone and adipose tissue. However, contrary to what was initially expected, plasma OC concentration was found to be lower in co-supplemented animals and positively associated with adipose mass. Plasma OC is considered a marker of bone formation [1,30], conferring beneficial effects on insulin sensitivity [31–33], and is therefore usually found to be negatively associated with body fat [5,34–36]. Unfortunately, additional determination of the under-carboxylated and active form of OC (uOC) did not contribute to shed

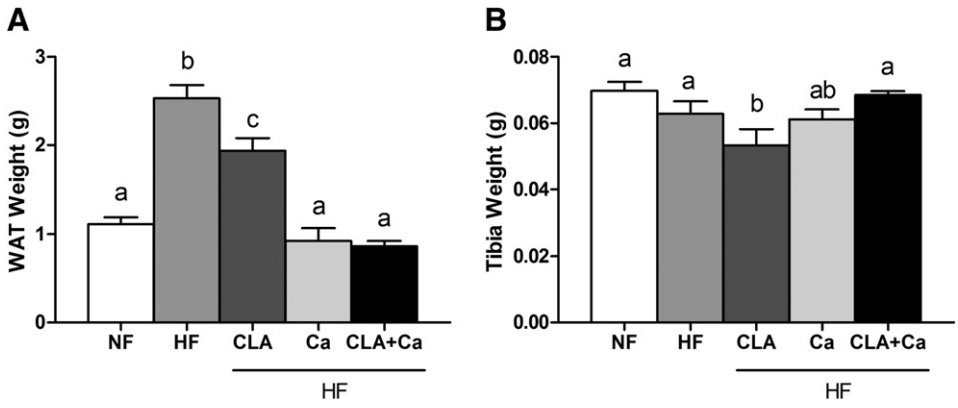

Fig. 1. WAT and tibia weight. (A) WAT (g) and (B) tibia (g) weights of animals were recorded at sacrifice, showing significant differences among groups. Data are the mean±S.E.M. of 7–8 animals/group. Letters indicate differences among groups; one-way ANOVA followed by a Bonferroni test (P<.05).

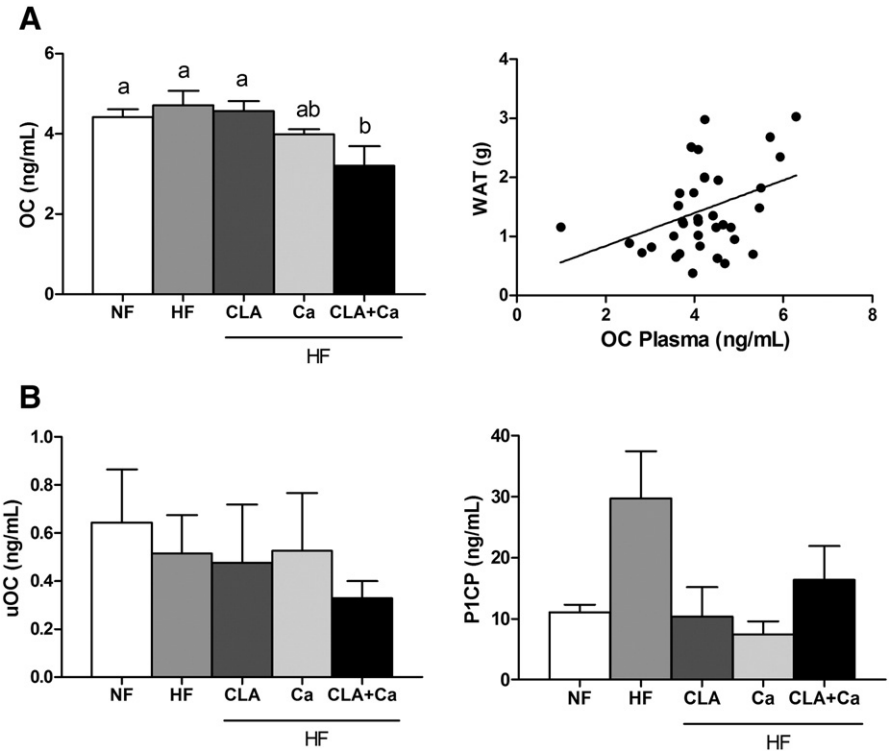

Fig. 2. Plasma biomarkers. (A) Plasma OC levels (ng/mL) were lower in CLA+Ca animals and correlated positively with WAT weight (g). (B) uOC and P1CP (ng/ml) showed no significant differences between groups. Data are the mean±S.E.M. of 6–8 animals/group. No differences among groups were found; one-way ANOVA followed by a Bonferroni test ($P<.05$).

more light on the role of OC. As stated in previous studies, this is a protein difficult to measure with precision [37,38]. Accordingly, a high variability between samples was observed, making it hard to reach to a specific conclusion as to the effects of CLA and calcium supplementation on the degree of active OC.

The use of CLA in the prevention of osteoporosis and bone mass loss has been extensively studied, but results are still inconsistent and there is no straightforward consensus on whether it promotes bone formation or resorption (discussed in review [39]). This inconsistency has been put down to various factors, sometimes associated with the nature of CLA. CLA is a term that designs a family of compounds which includes two main bioactive isomers, *cis*-9, *trans*-11 (9,11-CLA) and *trans*-10,*cis*-12 (10,12-CLA) CLA, which show different physiological properties [40]. 9,11-CLA diminishes osteoblast differentiation and increases adipocyte differentiation, whereas 10,12-CLA has an opposite effect [15,41]. Furthermore, when the isomers are given together, 10,12-CLA is responsible for the improvement of bone mass, in particular when given with calcium [17,18,42]. Decreased tibia weight in CLA animals, which was counteracted when given in co-supplementation with calcium, could be partially explained by the changes observed in skeleton gene expression. Both CLA (made up of both isomers) and calcium separately stimulated an increase in the expression of OST-PTP mRNA, a gene encoded by *Esp*, which is a negative regulator of OC secretion in bones and an inhibitor of OC decarboxylation [43]. Accordingly, bone OST-PTP expression was negatively correlated with tibia weight and positively associated with plasma glucose and HOMA index, associations which have also been previously described [4,44]. Furthermore, these data suggest that the expression of OST-PTP induced by CLA could explain the negative effect on glucose metabolism which has been previously described in this animal model using this dose of CLA [23]. However, calcium in co-supplementation with CLA was able to keep OST-PTP expression at control levels and was accompanied by induction of the expression of bone formation genes, in particular Bglap2 and Col1a1 [45]; as a result, these animals did not show reduced bone weight.

Moreover, the analysis of expression of energy metabolism-associated genes on tibia supports the feasibility that bone acts as an endocrine organ in a tight relationship with energy metabolism and is able to respond to dietary interventions. An impact was mainly observed on gene expression of key receptors in bone. Groups with higher leptin plasma levels [23] (HF and CLA animals) showed lower expression of Lepr on bone and presented lower tibia weight. Karsenty and Ferron [12,46] have done extensive work on this subject and have shown that leptin acts as an inhibitor of bone mass accrual, by reducing bone formation and increasing bone resorption. In agreement, calcium animals maintained plasma leptin at control levels [23], as well as bone Lepr expression and tibia weight. Moreover, Insr and Adipor1 were also induced by dietary calcium intake. Insr is a tyrosine kinase involved in the regulation of insulin signalling, particularly in skeletal muscle and adipose tissue [47], although it is expressed in a wide variety of cells such as osteoblasts [44]. Knock-out mice for the insulin receptor in osteoblasts are glucose intolerant and insulin resistant [14], supporting the hypothesis that correct insulin signalling in bone is necessary for glucose homeostasis in the organism. The increase observed in CLA+Ca animals of insulin receptor expression, particularly in comparison with CLA animals, is therefore of interest, since it would contribute to the improvement of glucose sensitivity seen in these animals [23].

Although adiponectin function is mainly associated to adipose tissue, the presence of the two receptors in osteoblasts, and evidence that it exerts its action in an autocrine/paracrine manner, suggest that adiponectin plays an important role in bone physiology in cross-talk with adipose tissue [48]. The increased expression of Adipor1 observed by calcium supplementation would fit with the induction seen on bone formation genes Bglap2 and Col1a1, contributing to the maintenance of bone mass.

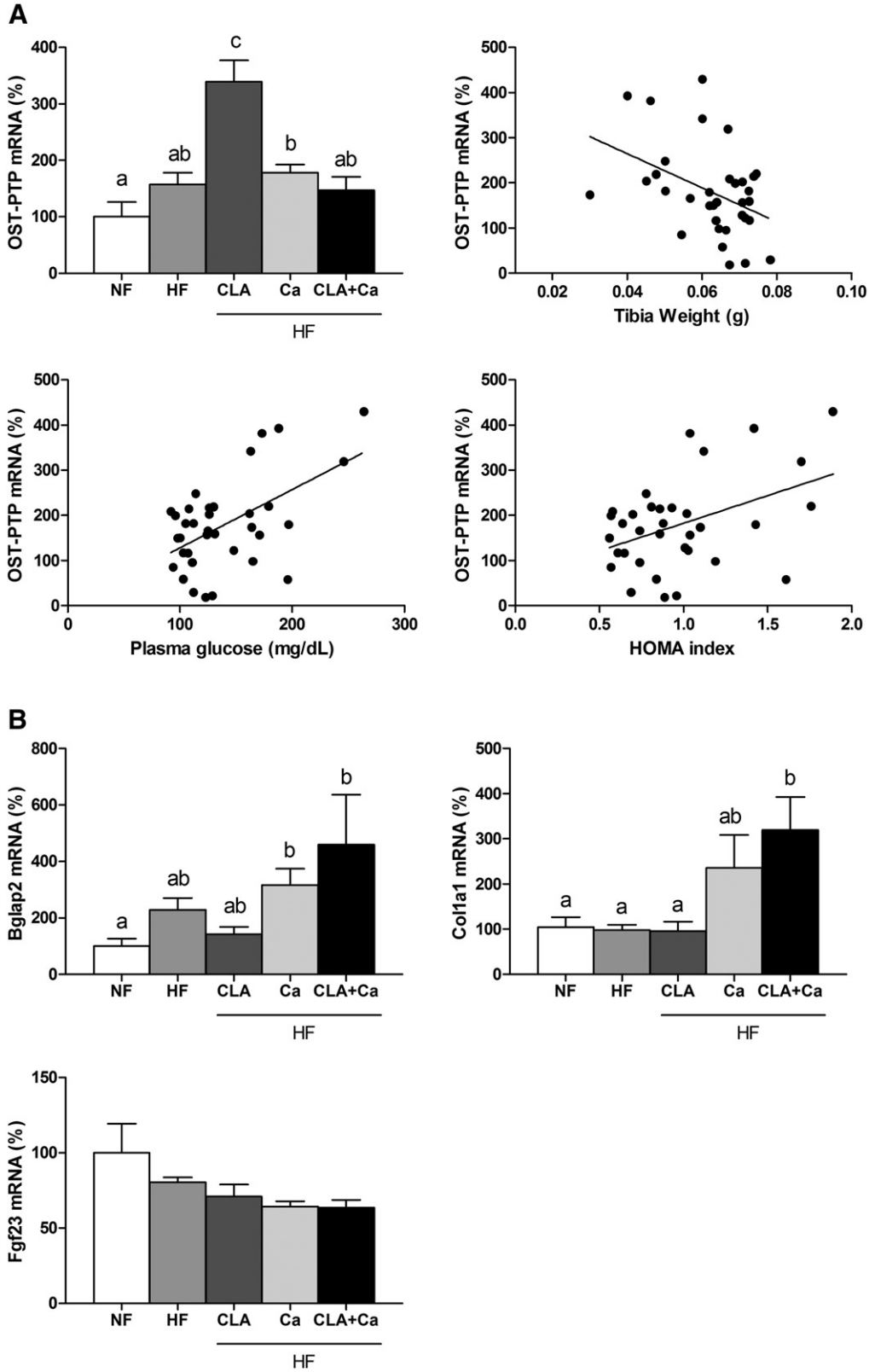

Fig. 3. Expression of bone (re)modeling genes in tibia. (A) OST-PTP mRNA (%) levels were found to be significantly higher in CLA and Ca animals. OST-PTP expression negatively correlated with tibia weight, whereas positive correlations were found with plasma glucose and HOMA index. (B) Bone gamma-carboxyglutamate protein 2 (Bglap2) and collagen, type I, alpha 1 (Col1a1) mRNA (%) expression was induced by co-supplementation, whereas no effects were seen on fibroblast growth factor 23 (Fgf23) mRNA. Data are the mean±S.E.M. of 6–8 animals/group. Letters indicate differences among groups; one-way ANOVA followed by a Bonferroni test ($P<.05$). Linear relationship between key variables was tested using Pearson's correlation coefficients ($P<.05$).

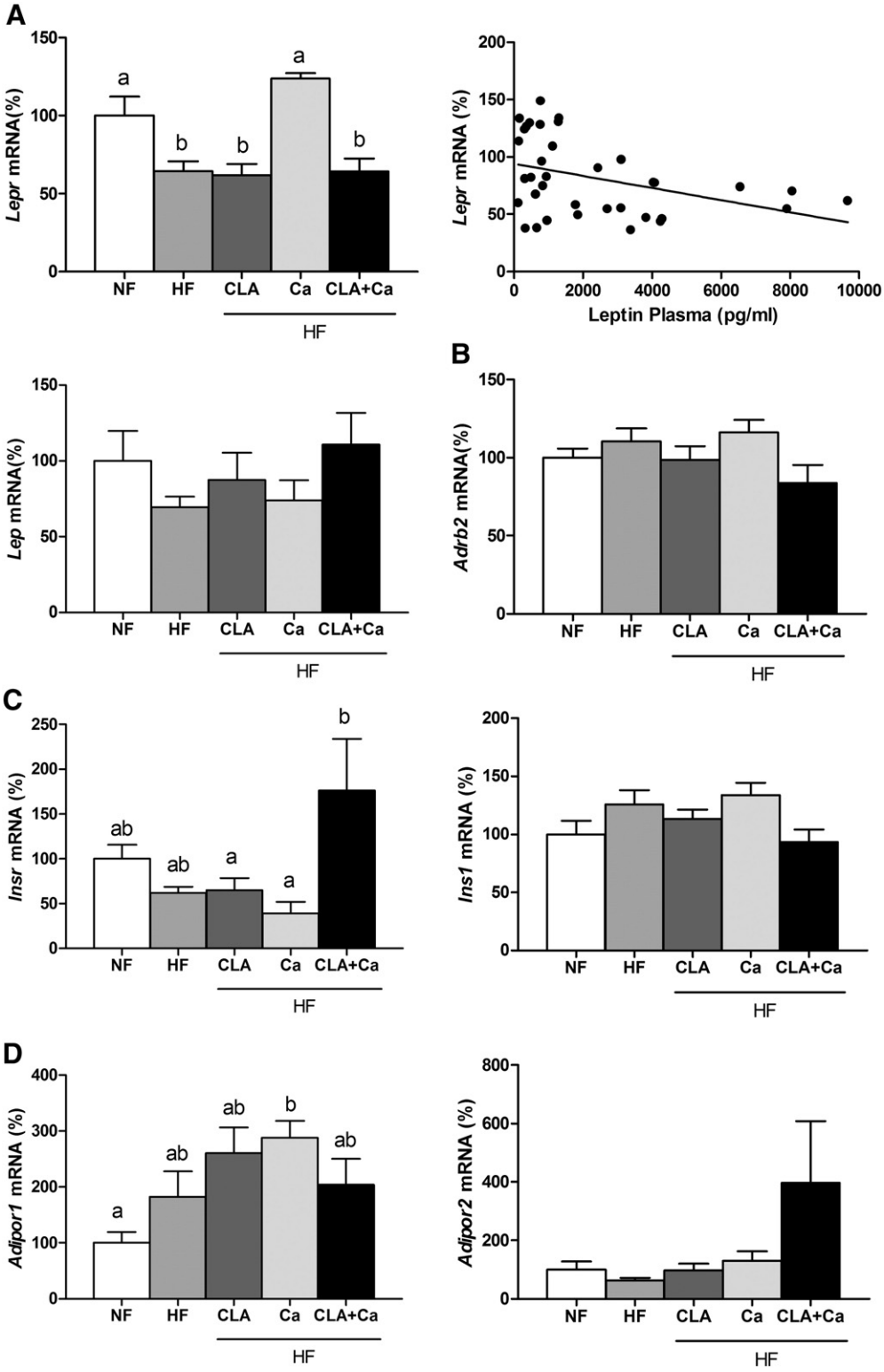

Fig. 4. Expression of energy metabolism associated genes in tibia. (A) Leptin receptor (Lepr) was lower in the HF, CLA and CLA+Ca groups, whereas calcium maintained control values. Moreover, bone Lepr mRNA negatively correlated with leptin in plasma. No differences were seen for leptin (Lep). (B) No effect was seen on adrenergic receptor beta 2 (Adrb2). (C) Insulin receptor (Insr) was increased in CLA+Ca animals, while no differences were seen for insulin (Ins1). (D) Adiponectin receptor 1 (Adipor1) was significantly increased in calcium-supplemented animals, and although adiponectin receptor 2 (Adipor2) levels were higher in CLA+Ca, this was not significant. Data are the mean±S.E.M. of 6–8 animals/group. Letters indicate differences among groups; one-way ANOVA followed by a Bonferroni test ($P<.05$). Linear relationship between key variables was tested using Pearson's correlation coefficients ($P<.05$).

Overall, we have shown that dietary supplementation with the two bioactive isomers of CLA and calcium, particularly co-supplemented, is able to modulate the expression of genes involved in bone (re)modeling and energy metabolism in tibia. Furthermore, interesting correlations are seen with key metabolism players, such as plasma leptin, suggesting an interaction with energy homeostasis and body fat

content in adult mice. Although further research is needed, this study highlights the beneficial effect of CLA in co-supplementation with calcium on bone health, supporting the fact that bone is able to respond to dietary inputs, and points out that strategies aiming to counteract obesity should take into account the potential cross-talk between adipose tissue and the skeleton.

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