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Dietary calcium attenuation of body fat gain during high-fat feeding in mice

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Abstract

Human epidemiological studies have supported the hypothesis that a dairy food-rich diet is associated with lower fat accumulation, although prospective studies and intervention trials are not so conclusive and contradictory data exist in animal models. The purpose of this study was to assess the effects on body weight and fat depots of dairy calcium (12 g/kg diet) in wild-type mice under ad libitum high-fat (43%) and normal-fat (12%) diets and to gain comprehension on the underlying mechanism of dairy calcium effects.

Our results show that calcium intake decreases body weight and body fat depot gain under high-fat diet and accelerates weight loss under normal-fat diet, without differences in food intake.

No differences in gene or protein expression of UCP1 in brown adipose tissue or UCP2 in white adipose tissue were found that could be related with calcium feeding, suggesting that calcium intake contributed to modulate body weight in wild-type mice by a mechanism that is not associated with activation of brown adipose tissue thermogenesis. UCP3 protein but not gene expression increased in muscle due to calcium feeding. In white adipose tissue there were effects of calcium intake decreasing the expression of proteins related to calcium signalling, in particular of stanniocalcin 2. CaSR levels could play a role in decreasing cytosolic calcium in adipocytes and, therefore, contribute to the diminution of fat accretion.

Results support the anti-obesity effect of dietary calcium in male mice and indicate that, at least at the time-point studied, activation of thermogenesis is not involved.

Keywords: Dietary calcium; Energy expenditure; Adipose tissue; Thermogenesis; UCP1; UCP2

1. Introduction

Epidemiological studies have shown a potential association between dairy food consumption and body weight regulation. In particular, human data from cross-sectional studies support the hypothesis that a dairy food-rich diet is associated with lower fat accumulation in both adults and children; although prospective studies and intervention trials have yielded nonconclusive results (for a recent review, see Ref. [1]). A great amount of work has been developed during recent years looking to provide evidence for a potential biological mechanism for this association. Using transgenic mice expressing agouti protein in adipose tissue under the control of the aP2 promoter, Zemel [2] demonstrated that high calcium diets reduce fat accretion and weight gain and increase thermogenesis in animals maintained at identical caloric intakes. However, no evidence for higher energy expenditure has been found in Wistar rats and the decrease in body weight and fat content have been at least partially attributed to faecal fat loss due to the formation of calcium soap [3]. Moreover, recent papers in C57BL/6J mice and also in Sprague-Dawley or Wistar rats do not support the hypothesis of dietary calcium in regulating energy metabolism and obesity [4,5]. Therefore, contradictory data exist concerning the effects of dietary calcium on body weight and energy metabolism in animal models. The aim of this work was to assess the effects on

Abbreviations: ANOVA, analysis of variance; au, arbitrary units; BAT, interscapular brown adipose tissue; C/EBPα, CCAAT enhancer binding protein α; CaSR, calcium sensing receptor; CPT-1b, carnitine palmitoyltransferase1b muscle; RARα, retinoid alpha receptor α; RXRα, retinoid X receptor α; SDS, sodium dodecyl sulphate; SSC, saline sodium citrate buffer; STC2, stanniocacin 2; UCP-1, UCP-2, UCP-3, uncoupling proteins 1, 2 and 3 isoforms; VDR, vitamin D receptor; WAT, white adipose depots.

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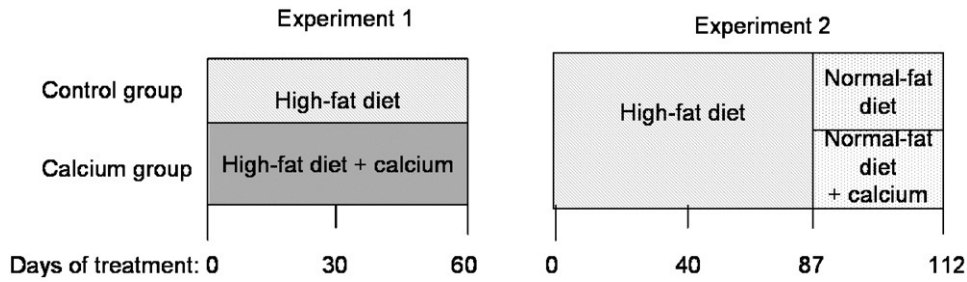

Fig. 1. Experimental design and dietary treatment followed by animals. In Experiment 1, male mice were fed a high-fat diet (control group) or the same diet supplemented with calcium (calcium group). In Experiment 2, animals were initially fed a high-fat diet (for 86 days) and then switched to a normal-fat diet either with normal (control group) or higher calcium content (calcium group).

body weight and body fat of dairy calcium in normal mice under high-fat and normal-fat diets and to gain comprehension about the potential involvement of thermogenic mechanisms, particularly in high-fat fed animals, which could also contribute to a better knowledge of the effect of calcium-rich diets in humans.

2. Methods and materials

2.1. Animals

Five-week-old male mice (C57BL/6J) from Charles River (Barcelona) were housed in groups of three in plastic cages, acclimated to 22°C with a 12-h light/12-h dark cycle and had free access to the diet and water. Animals were incorporated into one of the following experiments (see Fig. 1):

Experiment 1: Animals were fed ad libitum with a high-fat diet (43%) (control group, $n=6$) or with this diet supplemented with calcium (12 g/kg) (calcium group, $n=6$) for 2 months.

Experiment 2: Animals were fed ad libitum with a high-fat diet (43%) for 86 days to make them obese. Then, animals were fed with a standard, normal-fat, diet (12%) (control group, $n=6$) or with this normal-fat diet supplemented with calcium (calcium group, $n=6$) (12 g/kg) to the end of experiment.

All experimental procedures were performed according to the National and Institutional Guidelines for Animal Care and Use at the University of the Balearic Islands.

2.2. Diets

Diets were prepared by Research Diets Inc. (New Brunswick) and presented as pellets to the animals. The detailed diet composition is listed in Table 1. All diets provided 20% of calories from protein. In calcium diets, composition was aimed to supply 42% calcium from nonfat dry milk and the amount of purified casein was adjusted accordingly. The calcium content was 1.03 g/kcal in control diets and 3.10 g/kcal in the supplemented diets. Normal-fat diets provided 12% of calories from lard and soybean oil and were increased to 43% in high-fat diets. Carbohydrate was supplied as sucrose and cornstarch. The gross energy density of these formulations was calculated to be 3.8 kcal/g in normal-fat and 4.5 kcal/g in high-fat diets.

2.3. Determinations

2.3.1. Food intake and body weight

Food intake of animals was followed three times a week by weighing the amount of food left to the animals (per cage) and the remaining food on the following day. Experiment 1 was performed twice (the second time in parallel with Experiment 2). Body weight and food intake were available from 12 animals per group for Experiment 1 and from 6 animals per group for Experiment 2. Body weight was recorded for each individual animal at the same time as food intake.

Table 1
Diet composition in grams and expressed as a percentage of the calorie content

Diet composition	High fat		Normal fat	
	Control	Calcium	Control	Calcium
Ingredient (g)				
Casein, 80 mesh	212	50	214	50
DL-Methionine	3	3	3	3
Sucrose	350	142	400	439
Corn starch	0	0	250	0
Cellulose	50	50	50	50
Soybean oil	66	62	32	30
Lard	120	120	20	20
t-Butylhydroquinone	0.014	0.014	0.014	0.014
Mineral mix S10022B	7	7	7	7
Calcium carbonate	10	17.4	10	17.4
Potassium phosphate, monobasic	8	0	8	0
Potassium citrate, 1 H_2O	1.6	1.6	1.6	1.6
Vitamin mix V10037	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Milk, nonfat, dry	0	400	0	400
kcal (%)				
Protein	19.6	19.8	19.8	19.8
Carbohydrate	37.2	37.2	68.2	67.8
Fat	43.2	43.0	12.1	12.4
kcal/g	4.61	4.48	3.84	3.76
Calcium content (mg/kcal)	1.03	3.10	1.03	3.10

2.3.2. Tissue sampling and determinations

At the end of each experiment, animals were sacrificed and liver, kidneys, muscle, interscapular brown adipose tissue (BAT) and white adipose depots (WAT) were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at −70°C.

Total RNA and protein was extracted from tissue samples from six animals of Experiment 1 using Tripure reagent (Roche, Barcelona, Spain) according to the instructions provided by the supplier. Protein concentration was determined by the BCA assay (Pierce) using a BSA standard supplied with the kit.

2.3.3. Western blots

UCP-1, UCP-2, UCP-3, C/EBPα, retinoid X receptor (RXRα), retinoid alpha receptor (RARα), vitamin D receptor (VDR) and calcium sensing receptor (CaSR) were determined by Western blotting. β-Actin was determined in some gels to confirm equal protein load charge between samples.

The procedure was as follows: 30–100 μg of protein was heat denatured in sample buffer [62.5 mM Tris, 5% sodium dodecyl sulphate (SDS), 10% v/v glycerol, 5% v/v β-mercaptoethanol, 0.0025% bromophenol blue] and electrophoresed on 10% SDS-polyacrylamide gel electrophoresis according to Laemmli as previously described [6]. Proteins were then electrotransfered (semi-dry electroblotter) to nitrocellulose membrane 0.45 μm (Bio-Rad, Madrid, Spain), and Ponceau-S (0.1% in 5% acetic acid; Sigma) staining was performed to confirm equal loading/transfer. Following transfer, membranes were blocked with 5% or 1% milk in PBS-Tween 20 for 1 h or overnight, respectively. Then they were incubated with the primary antibody with 0.1% BSA in PBS-Tween 20 for 1 h. The primary antibodies used were rabbit anti-mouse UCP-1 (1:1000–1:3000, Alpha Diagnostic International); rabbit anti-mouse UCP-2 (1:1000, Alpha Diagnostic International); guinea pig anti-human UCP-3 (1:5000, Linco Research, Inc); rabbit polyclonal anti-rat C/EBPα (1:4000, Santa Cruz Biotech); rabbit polyclonal anti-human RXRα (1:2000–1:3000, Santa Cruz Biotech); rabbit polyclonal anti-human RARα (1:200–1:1000, Santa Cruz Biotech); rat monoclonal anti-VDR (1:1300, Sigma); rabbit polyclonal anti-rat CaSR (1:100–1:1000, Abcam); and anti-β actin mouse monoclonal antibody (1:1000, Abcam). Membranes were then washed with PBS-Tween 20 and incubated with the secondary peroxidase-linked anti-rat IgG or anti-rabbit IgG antibody (Amershan Biosciences, Barcelona, Spain) or anti-guinea pig (Linco Research) diluted 1:5000 with 0.1% BSA in PBS-Tween 20. The immunocomplexes were revealed using an enhanced chemiluminescence detection system (ECL, Amershan Biosciences, Barcelona, Spain) and visualized by exposure to sensitive films (Hyperfilm ECL, Amershan Biosciences). The films were scanned in ChemiGenius (SynGene) using the software GeneSnap (6.03 version), and the bands were quantified using GeneTools version 3.04 (SynGene).

2.3.4. Northern blots

Twenty- to 30 μg of total RNA, denatured with formamide/formaldehyde, was fractionated by agarose gel electrophoresis as previously described [7]. The RNA was then transferred onto a Hybond Nylon membrane in 20× SSC (saline sodium citrate buffer: 1× SSC in 150 mM NaCl, 15 mM sodium citrate, pH 7.0) by capillary blotting for 16 h and fixed with UV light [7]. The specific mRNAs were detected by a chemiluminescence-based procedure, using antisense oligonucleotide probes (Table 2) synthesised commercially (TIB MOLBIOL) and labelled at both ends with a single digoxigenin ligand. Prehybridization was at 42°C for 15 min in DIG-Easy Hyb (Roche). Hybridization was at 42°C overnight in DIG-Easy Hyb containing 35 ng/ml of the oligonucleotide probe. Then, hybridized membranes were washed twice for 15 min at room temperature with 2× SSC/0.1% SDS, followed by two 15-min washes at 48°C with 0.1× SSC/0.1% SDS. After 1-h blocking at room temperature with a blocking reagent (Roche), the membranes were incubated first with antidigoxigenin-alkaline phosphatase conjugate (Roche) and then with the chemiluminescent substrate CDP-Star (Roche). Finally, membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). The films were scanned and quantified as described for the Western films. Finally, blots were stripped by 10-min exposure to boiling 0.1% SDS and re-probed for other mRNA or finally for 18S rRNA detection, to check the loading and transfer of RNA during the blotting.

2.3.5. Reverse transcriptase–polymerase chain reaction analysis of carnitine palmitoyltransferase1b muscle mRNA

Carnitine palmitoyltransferase1b muscle (CPT-1b) mRNA expression was assessed by reverse transcription–PCR assays using β-actin expression as an internal control in muscle. In brief, 1 μg of total RNA was denatured at 65°C for 10 min and reverse transcribed to cDNA using MuLV reverse transcriptase (according to Applied Biosystems's procedure) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in a Perkin Elmer 2400 Thermal Cycler (Norwalk, CT, USA). Two microlitres of the RT product was used for PCR amplification following the "hot

Table 2
Probes used for Northern blot hybridization

Probe	Gene
5′-GTTGGTTTTATTCGTGGTCTCCCAGCATAG-3	UCP-1
5′-GGCAGAGTTCATGTATCTCGTCTTGACCAC-3′	UCP-2
5′-GACTCCTTCTTCCCTGGCGATGGTTCTGTAGG-3	UCP-3
5′-TCCCACGAGCCACAGGCAGAGCCACAGGAGCAGC-3′	Resistin
5′-GGTCTGAGGCAGGGAGCAGCTCTTGGAGAAGGC-3′	Leptin
5′-CCCTCGCTCACCCTTGGCACCTCTGTTGGC-3	STC-2
5′-CTCGATAATGTCAGCCATCGCGGTGGCCTG-3′	CaSR
5′CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3′	18S rRNA

start PCR" method and using the TAQ DNA polymerase in buffer B (Promega, Barcelona, Spain). The sample was first denatured at 95°C for 105 s and then PCR was carried out using the following parameters: 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. Twenty-two cycles were used for both CPT-1 and β-actin. The amplification was finished by a final extension step of 7 min at 72°C. Primers for the CPT-1b gene were as follows: f5′ -AAGGGTAGAGTGGG-CAGAGG -3′ and r5′-GCAGGAGATAAGGGT-GAAAGA-3′, and for the β-actin gene were as follows: f5′-GTGGTGGTGAAGCTGTAGCC-3′ and r5′-ACGGGCATTGTGATGGACTC-3′. The expected size of the products was 222 bp for the CPT-1b gene and 165 bp for the β-actin gene, which were visualized by electrophoresis in a 2% agarose gel containing ethidium bromide and verified by using a DNA 100-bp ladder. The bands in the gel were quantified as described above. The signal for CPT-1b mRNA was normalized to the signal of the housekeeping gene β-actin, and the results were expressed as the CPT-1b/β-actin mRNA ratio.

2.4. Statistics

Data are presented as mean values±S.E.M. Differences between groups were assessed by two-way analysis of

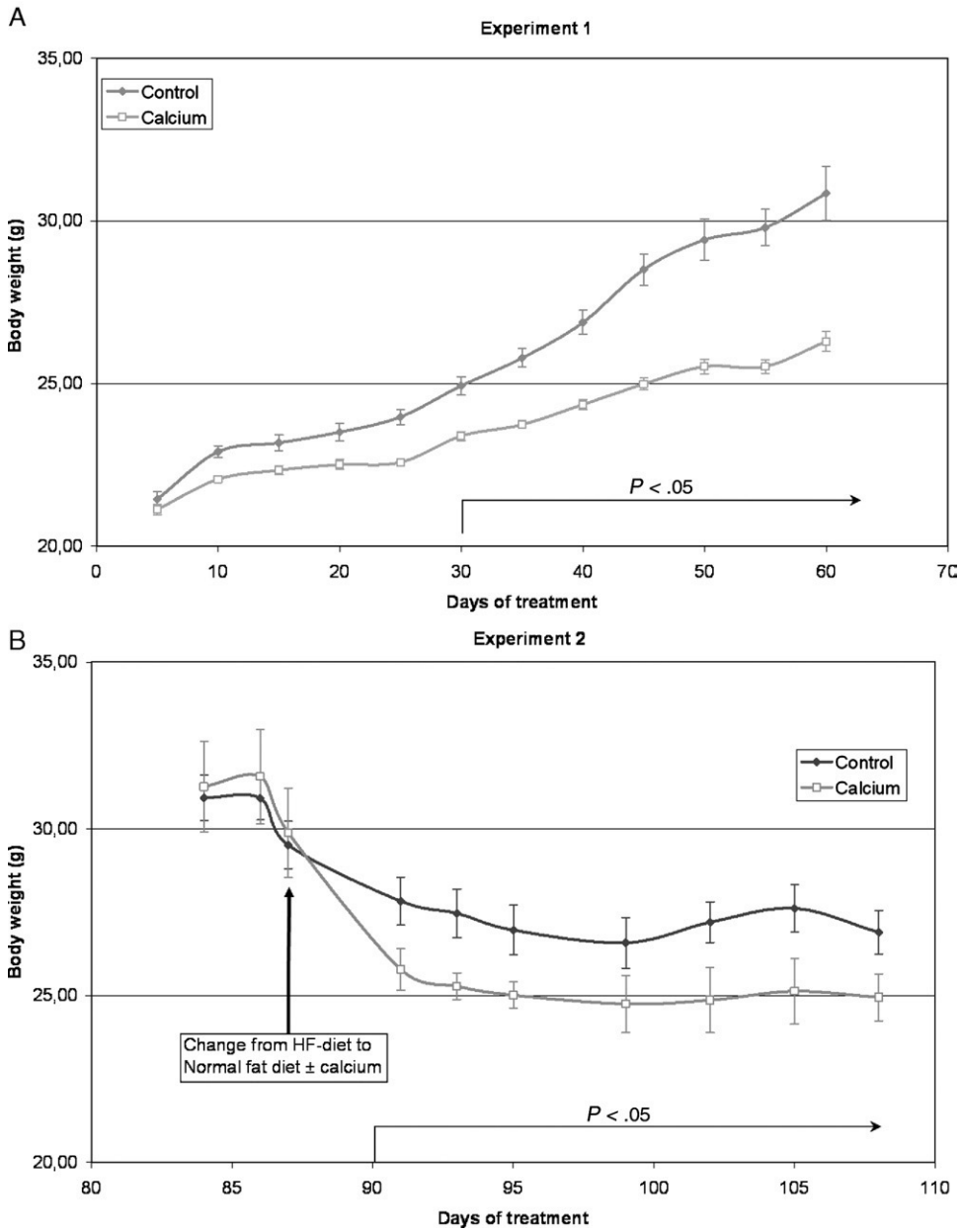

Fig. 2. Body weight evolution of animals from Experiments 1 (A) and 2 (B) during dietary treatment. In (A), animals were fed ad libitum a high-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium). In (B), after 86 days of high-fat feeding, animals were switched to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) ($n=12$ for Experiment 1 and $n=6$ for Experiment 2). Body weights were significantly different between groups from Day 30 onwards (ANOVA) in (A) and from Day 90 onwards (ANOVA) in (B).

Table 3
Body and tissue weights in mice at the end of dietary treatments

Diet	Experiment 1 ($n=12$)		Experiment 2 ($n=6$)	
Group	High fat		Normal fat	
	Control	Calcium	Control	Calcium
Body weight (g)	31.1±1.0	26.8±0.4*	27.1±0.5	26.4±0.5
Liver (g)	1.28±0.03	1.18±0.03*	1.03±0.09	1.17±0.1
Kidneys (mg)	356±9	357±11	369±2	361±2
White adipose (g)				
Inguinal	0.822±0.07	0.305±0.02*	0.373±0.04	0.389±0.04
Epididymal	1.26±0.11	0.584±0.05*	0.580±0.07	0.585±0.04
Mesenteric	0.689±0.08	0.456±0.03*	0.507±0.04	0.532±0.04
Retroperitoneal	0.492±0.047	0.179±0.022*	0.182±0.03	0.155±0.008
Sum	*3.27±0.3*	*1.52±0.1**	*1.64±0.1*	*1.66±0.09*
Brown adipose (mg)	203±17	109±6*	158±5	143± 1

Statistically significant differences between groups were determined by Student's t test (*$P<.05$).

variance (ANOVA) or by Student's t-test with the level of significance set at $P<.05$. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL, USA).

Data obtained by Western and Northern blot have been referred to the values of control animals and are expressed in arbitrary units (au) with respect to milligrams of total tissue protein or total RNA, respectively.

3. Results

3.1. Body and tissue weights and food intake

3.1.1. Experiment 1

Although food consumption did not differ between groups (11.3±0.4 kcal/day in the control group and 11.4±0.4 kcal/day in the calcium group), the calcium group showed a lower rate of body weight gain (Fig. 2A) during the period of high-fat feeding. The amount of food eaten elicited a calcium intake of 11.6±0.4 mg Ca/day in control animals and 34.2±1.1 mg Ca/day in calcium-fed animals ($P<.05$). Body weight differences were statistically significant from Day 30 onwards ($P<.05$), and, at the end of the treatment, body weight in the calcium group was 14% lower than in controls ($P<.05$) (Table 3). All adipose tissue depots were significantly reduced in high-calcium-fed animals (Table 3). In fact, the sum of fat pads decreased by half; mesenteric was the least affected, showing a decrease of 34% in weight, and the greatest decrease was seen in the retroperitoneal fat pad (64%). Interscapular brown fat depot (BAT) followed a similar trend, being 46% lower in calcium-fed animals than in controls. Kidney weight was not affected by the treatment, and liver weight was slightly but significantly lower ($P<.05$) in calcium-fed animals than in control animals (Table 3).

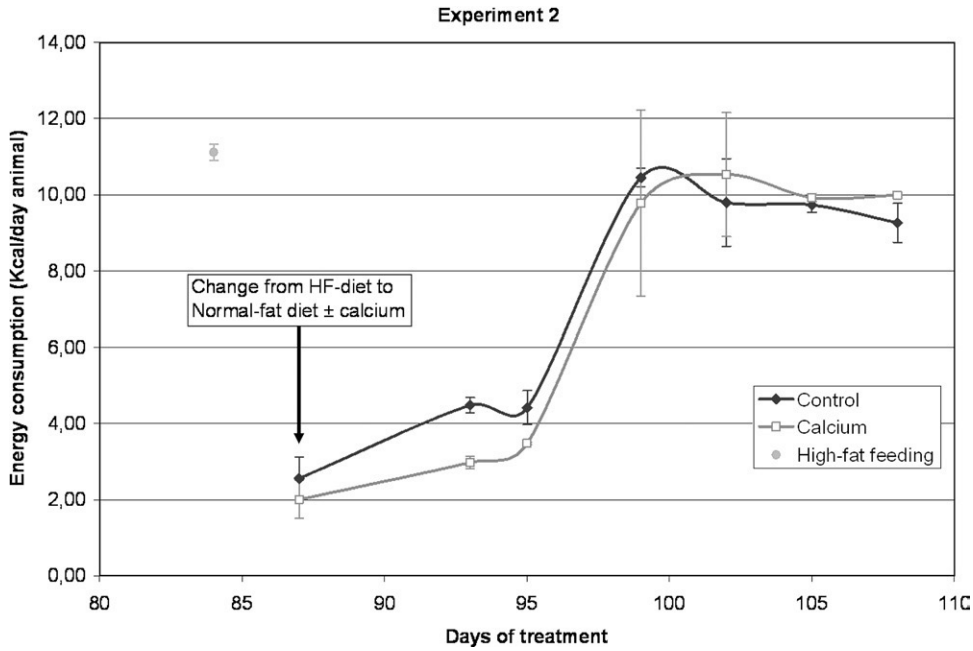

Fig. 3. Energy intake in animals from Experiment 2, fed on a high-fat diet and switched (at Day 86) to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) ($n=6$ animals per group).

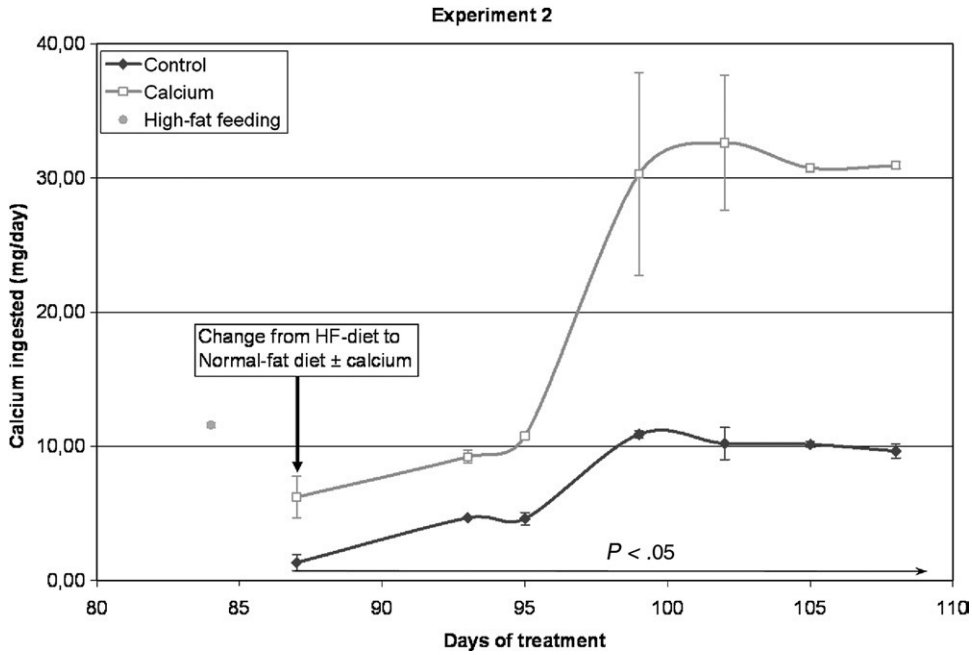

Fig. 4. Calcium intake in animals from Experiment 2, fed on a high-fat diet and switched (at Day 86) to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) ($n=6$ animals per group). Calcium intake was significantly different between groups from Day 87 onwards (ANOVA).

3.1.2. Experiment 2

During the period of high-fat feeding, food intake did not differ between the groups and provided 11.1±0.2 kcal/day and 11.6±0.2 mg Ca/day on average. Substitution of the high-fat diet by the normal-fat diet was accompanied by a drastic reduction in food intake in both groups and took around 14 days to reach a steady level (Fig. 3). Once food intake was stabilised, there were no significant differences between the groups in terms of calorie content (C: 10.1±0.2 kcal/day; Ca: 9.9±0.4 kcal/day) from Day 100 onwards. However, in calcium-fed animals, the calcium intake was higher from the first day, they achieved the previous calcium intake level faster and their calcium level rose to a steady level three times higher than that of controls. By the end of the experiment, calcium intake was stabilised at 10.6±0.4 mg Ca/day in controls and 30.8±0.8 mg Ca/day ($P<.05$) in calcium animals (Fig. 4).

3.2. Tissue gene and protein expression

3.2.1. Brown adipose tissue

Feeding animals with a high-calcium diet (Experiment 1) was accompanied by a drastic reduction in the mRNA expression of leptin in brown adipose tissue (Table 4). UCP1 showed a nonstatistically significant reduction in both protein and mRNA expression (61% and 52%, respectively). No changes were observed in the expression of RXRα, C/EBPα, VDR or resistin. By contrast, RARα expression showed increased levels in calcium-fed animals (Table 4).

3.2.2. Muscle

UCP3 mRNA levels were not different between groups (100±15, control; 68±13, calcium), but protein levels were increased by 63% ($P<.05$) in calcium-fed animals. CPT-1b mRNA levels were not different between groups (100±4, control; 83±7, calcium). No differences were observed in protein levels of VDR (100±6, control; 96±7, calcium), RARα (100±8, control; 114±5, calcium), RXRα (100±30 control; 96±9, calcium) or CaSR (100±11, control; 93±7, calcium) by calcium diet.

3.2.3. White adipose

Protein expression of VDR (100±35, control; 106±13, calcium), resistin (100±14, control; 108±29, calcium) and UCP2 (100±20, control; 92±15, calcium) was not different between groups. The same was seen for UCP2

Table 4
Protein and RNA expression of target factors in brown adipose tissue from animals fed on a high-fat diet (control) and supplemented with calcium (calcium)

Brown adipose	High-fat diet	
	Control	Calcium
Protein (au/mg protein)		
VDR	100±13	103±7
RARα	100±12	176±24*
RXRα	100±19	115±22
C/EBPα	100±13	112±16
UCP1	100±21	61±9
mRNA (au/mg RNA)		
UCP1	100±24	52±18
Resistin	100±30	117±9
Leptin	537±18	Not detected*

Data have been referred to the values of control animals (set at 100%) and are expressed in arbitrary units with respect to milligrams of total tissue protein or total RNA.
$n=6$ animals for each group. Statistically significant differences between groups were determined by Student's t test (*$P<.05$).

mRNA (100±13, control; 82±7, calcium). Levels of C/EBPα (100±32, control; 54±17, calcium) and CaSR proteins (100±9, control; 56±11, calcium) and leptin mRNA (100±23, control; 59±8, calcium) showed a decrease in calcium-fed animals, but did not attain statistical significance. Staniocalcin-2 mRNA was decreased in calcium-fed animals (100±10, control; 68±7, calcium; $P<.05$).

4. Discussion

4.1. Body weight and food intake

Our results support the hypothesis that calcium intake contributes to combat obesity in mice. High-fat diet produces an increase in body weight that is counteracted by feeding ad libitum a high-calcium diet from a dairy source (42% of calcium from milk). The anti-obesity effect was particularly seen during the weight-gain phase (Experiment 1) as has been described in other animal models [2–4,8]. Furthermore, during the slimming phase (Experiment 2), calcium supplementation also played a role against obesity, contributing to a faster rate of body weight loss.

Our results indicate that no aversion to food is present; calcium animals are eating the same amount of food as the corresponding controls, irrespective of calcium or fat content in the diet. Probably because we are using moderately high calcium diets (0.4% in control and 1.2% in calcium-fed animals) and from a dairy source, which seems to be the best source concerning this aspect [9]. It is remarkable that there was a drastic decrease in food intake observed when the high-fat diet was replaced with a normal-fat diet (Experiment 2, Fig. 3). After eating a palatable diet, animals do not like the not-so-tasty normal-fat diet [10]. Therefore, the decrease in food intake seems to be the major factor responsible for the initial reduction in body weight seen in both groups (control and calcium) after changing the dietary regime. Since no differences in calorie load were found, it is suggestive to assume that higher calcium intake played a role in the accelerated body weight loss seen in the high-calcium group.

In Experiment 1, calcium intake was accompanied by lower liver weight. This has also been found in rats fed a high-calcium diet [3] and actually reflects lower body fat content, as adipose fat pads were reduced by 53%. Interestingly, the fat content in high-fat, high-calcium-fed animals (Experiment 1) was similar to that found in both groups of animals from Experiment 2 (control and calcium-fed animals), suggesting the presence of underlying mechanisms directed to modulate fat content close to 'control' values.

Altogether, these results support the anti-obesity effect of dietary calcium in male mice. Dairy calcium contributed to the attenuation of body weight and fat gain during high-fat feeding and also to the faster slimming rate in animals switched from a high-fat diet to a normal-fat one under ad libitum conditions.

To gain insight into the molecular mechanism involved in the decrease of fat accretion by dietary calcium, particularly during the high-fat feeding (Experiment 1), we first tested the hypothesis that uncoupling proteins could be involved in participation in activation of adaptive thermogenesis.

UCP1 is expressed in brown adipocytes and constitutes the main thermogenic effector in small rodents (for review, see Ref. [11]). UCP1 levels, assessed at the level of transcription and translation, were not different between calcium and control animals (Table 4). In fact, we found a decrease in UCP1 expression (61% in protein and 52% in mRNA levels), although animal variability and/or sample size did not sustain statistical significance between calcium and control animals. Furthermore, the decrease in UCP1 expression correlates with the increased levels of RARα as we and others have previously described, that UCP1 induction goes in parallel with a decrease in RARα during BAT differentiation [12,13]. Therefore, our data did not indicate an activation of thermogenesis in BAT, but a decreased diet-induced thermogenesis in high-calcium-fed animals.

In addition to UCP1, other uncoupling proteins such UCP2 and UCP3 may also act as uncouplers of oxidative phosphorylation and, therefore, contribute to modulating metabolic efficiency. In contrast to UCP1, UCP3 is predominantly expressed in skeletal muscle, and although it needs further assessment, its primary physiological role may be involved in mitochondrial handling of fatty acids [14]. Our data showed increased UCP3 protein levels (but not mRNA) in high-calcium-fed animals, which could have a role in protecting mitochondria against fatty acid accumulation and might help to maintain muscular fat oxidative capacity. However, CPT-1b expression, which is a key factor in mitochondrial fatty acid oxidation, did not indicate a higher rate of fat oxidation in calcium-fed animals.

Concerning modulation of UCP2, our data do not indicate an activation of its transcription in WAT as has been seen in aP2-agouti transgenic mice fed on a high-dietary calcium diet which shows increased UPC3 transcription in muscle too [15]. These activations have been linked to the reduction found in ROS production by high-calcium diet feeding, indicating a role of mitochondrial uncoupling in counteracting oxidative stress. However, our data on uncoupling proteins suggest high-calcium feeding in wild-type mice is not accompanied by activation of thermogenesis, maybe because there is no need to counteract oxidative stress as previously seen in this animal model [15] and it is closer to the trend of inhibition of thermogenesis found in other animal models of dietary obesity [16].

Gene expression parameters related to adipose tissue metabolism (C/EBPα, resistin, leptin) and/or calcium signalling (STC-2, VDR, CaSR) were measured in adipose tissue. C/EBPα is the initiation marker of the signalling cascade for adipocyte differentiation, responsible for the expression of adipocyte-specific genes and is expressed late in the differentiation program [17,18]. Our data show that C/

EBPα is not significantly altered by dietary calcium either in BAT or in WAT.

Resistin is expressed in brown and white adipocytes [19,20] and participates in the regulation of energy homeostasis (see Ref. [21] for review). At least in rodents, resistin plays an important role in the development of insulin resistance (see Ref. [22] for review). In this paper, we have shown that the expression of resistin in brown and white adipocytes is not affected by dietary calcium intake and/or body fat content. Therefore, the slimming effect of dietary calcium under high-fat feeding does not contribute to impair insulin signalling as has been observed with other nutrients such as CLA in particular conditions [23].

Leptin, an important signalling factor in obesity, shows a pattern well correlated with the diminution of body fat stores in high-calcium animals, and the disappearance of its expression in BAT is remarkable.

We studied the expression of STC2, which shares limited sequence similarity with an antihypercalcemic hormone first discovered in fish [24,25] and is widely expressed in mammals [26]. There is only limited information available on STC2 expression and function, but a role in mammal calcium homeostasis has been proposed [26], and, recently, a novel function for STC2 in protecting neuronal cells from oxidative stress and hypoxia has also been reported [27]. Although the exact function of STC2 in adipose cells has not been previously studied, whether this could be related to oxidative stress, the reduction found in its expression in WAT of high-calcium-fed animals is well correlated with the pattern of UCP2 shown in these animals. Therefore, these data also support the fact that induction of oxidative stress is not present in adipose cells of wild-type mice fed a high-fat-enriched calcium diet.

VDR is a transcription factor belonging to the superfamily of steroid/thyroid hormone receptors. Traditionally associated with calcemic activities, VDR is also known to be involved in cell proliferation, differentiation and immunomodulation using RXR as obligate partner (see Ref. [28] for review) and, recently, a role in modulating adipogenesis has been associated with VDR [29]. The data do not show any difference in adipose tissues or in muscle of high-calcium-fed mice. Therefore, the loss of fat accretion in high-calcium-fed animals does not seem to be modulated by a mechanism involving VDR, at least in the tissues studied.

CaSR is a G protein-coupled receptor that was originally thought to be mainly involved in calcium homeostasis, as the primary regulator of PTH secretion in response to changes in circulating calcium [30]. We found lower PTH serum levels in high-calcium-fed animals (data not shown), which are in agreement with higher levels of extracellular calcium that would inhibit parathyroid hormone secretion [31]. On other hand, several studies mainly made by the group of Zemel [32] have already demonstrated that increased intracellular calcium in adipocytes results in stimulation of lipogenic gene expression and suppression of lipolysis, which achieve adipocyte lipid filling and increased adiposity. Therefore, activation of CaSR in adipocytes could mediate an increase in intracellular calcium that could be responsible for triggering signalling cascades that would influence adipogenesis and triglyceride storage in fat cells. Our data demonstrate expression of CaSR in WAT and muscle of mice. No activation of CaSR was found in adipose tissue (if there was any effect, there is a nonstatistically significant reduction of its expression), suggesting that no increased cytosolic calcium is present in adipocytes, in accordance with data from other high-calcium-fed animal models [15,33]. The molecular basis involving the decrease in intracellular calcium could be related to CaSR, as antagonists used in other cell types have been shown to mimic this effect [34]. Altogether, our data suggest that modulation of CaSR in adipocytes could attenuate the intracellular calcium levels and, thus, contribute to the pattern of reduced fat accretion seen in high-calcium-fed animals.

It can be concluded that dietary calcium has a role in modulating body weight in wild-type male mice. Dairy calcium contributed to the attenuation of body weight gain during high-fat feeding. Furthermore, a faster slimming rate was also observed when animals were switched from a high-fat diet to a normal-fat one high in calcium content under ad libitum conditions. However, the effect of calcium on body weight is not due to a decrease in food intake; neither can it be explained by activation of BAT thermogenesis. The known effect of dietary calcium decreasing intestinal bioavailability of fat [3] may contribute to counteract any effect of calcium on thermogenesis. CaSR levels could play a role in decreasing cytosolic calcium in adipocytes and therefore contribute to the diminution of fat accretion. No evidence of oxidative stress is present in this animal model and the role of STC-2 in adipocytes, particularly in this aspect, would need further research. Finally, our data point out that wild-type mice do not follow the metabolic adaptations found in the aP2-agouti transgenic model under high-fat, high-calcium feeding and indicate that activation of uncoupling proteins does not seem to be the main factor responsible for the slimming effect of high-calcium diets.

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References

[1] Barba G, Russo P. Dairy foods, dietary calcium and obesity: a short review of the evidence. Nutr Metab Cardiovasc Dis 2006;16:445–51.
[2] Zemel MB. Role of dietary calcium and dairy products in modulating adiposity. Lipids 2003;38:139–46.
[3] Papakonstantinou E, Flatt WP, Huth PJ, Harris RB. High dietary calcium reduces body fat content, digestibility of fat, and serum vitamin D in rats. Obes Res 2003;11:387–94.

[4] Zhang Q, Tordoff MG. No effect of dietary calcium on body weight of lean and obese mice and rats. Am J Physiol Regul Integr Comp Physiol 2004;286:R669–77.
[5] Paradis S, Cabanac M. Calcium deficiency cannot induce obesity in rats. Physiol Behav 2005;85:259–64.
[6] Ribot J, Oliver P, Serra F, Palou A. Retinoic acid modulates the retinoblastoma protein during adipocyte terminal differentiation. Biochim Biophys Acta 2005;1740:249–57.
[7] Oliver P, Pico C, Martinez N, Bonet ML, Palou A. In vivo effects of CGP-12177 on the expression of leptin and uncoupling protein genes in mouse brown and white adipose tissues. Int J Obes Relat Metab Disord 2000;24:423–8.
[8] Shi H, Dirienzo D, Zemel MB. Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted aP2-agouti transgenic mice. Faseb J 2001;15:291–3.
[9] Zemel MB, Miller SL. Dietary calcium and dairy modulation of adiposity and obesity risk. Nutr Rev 2004;62:125–31.
[10] Proenza AM, Llado I, Serra F, Pico C, Pons A, Palou A. Tissue composition in persistent dietary obesity after early and adulthood overfeeding in the rat. Arch Int Physiol Biochim Biophys 1992;100: 147–54.
[11] Rousset S, Alves-Guerra MC, Mozo J, Miroux B, Cassard-Doulcier AM, Bouillaud F, et al. The biology of mitochondrial uncoupling proteins. Diabetes 2004;53(Suppl 1):S130–5.
[12] Bonet ML, Puigserver P, Serra F, Ribot J, Vazquez F, Pico C, et al. Retinoic acid modulates retinoid X receptor alpha and retinoic acid receptor alpha levels of cultured brown adipocytes. FEBS Lett 1997; 406:196–200.
[13] Alvarez R, Checa M, Brun S, Vinas O, Mampel T, Iglesias R, et al. Both retinoic-acid-receptor- and retinoid-X-receptor-dependent signalling pathways mediate the induction of the brown-adipose-tissue-uncoupling-protein-1 gene by retinoids. Biochem J 2000;345(Pt 1): 91–7.
[14] Krauss S, Zhang CY, Lowell BB. The mitochondrial uncoupling-protein homologues. Natl Rev Mol Cell Biol 2005;6:248–61.
[15] Sun X, Zemel MB. Dietary calcium regulates ROS production in aP2-agouti transgenic mice on high-fat/high-sucrose diets. Int J Obes (Lond) 2006;30:1341–6.
[16] Puigserver P, Llado I, Palou A, Gianotti M. Evidence for masking of brown adipose tissue mitochondrial GDP-binding sites in response to fasting in rats made obese by dietary manipulation. Effects of reversion to standard diet. Biochem J 1991;279(Pt 2):575–9.
[17] Rangwala SM, Lazar MA. Transcriptional control of adipogenesis. Annu Rev Nutr 2000;20:535–59.
[18] Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol 2000;16:145–71.
[19] Oliver P, Picó C, Serra F, Palou A. Resistin expression in different adipose tissue depots during rat development. Mol Cell Biochem 2003;252:397–400.
[20] Viengchareun S, Zennaro MC, Pascual-Le Tallec L, Lombes M. Brown adipocytes are novel sites of expression and regulation of adiponectin and resistin. FEBS Lett 2002;532:345–50.
[21] Rajala MW, Scherer PE. Minireview: the adipocyte-at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 2003;144:3765–73.
[22] Haluzik M, Haluzikova D. The role of resistin in obesity-induced insulin resistance. Curr Opin Investig Drugs 2006;7:306–11.
[23] Taylor CG, Zahradka P. Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. Am J Clin Nutr 2004;79: 1164S–8S.
[24] Wagner GF, Hampong M, Park CM, Copp DH. Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol 1986;63:481–91.
[25] Butkus A, Roche PJ, Fernley RT, Haralambidis J, Penschow JD, Ryan GB, et al. Purification and cloning of a corpuscles of Stannius protein from *Anguilla australis*. Mol Cell Endocrinol 1987;54:123–33.
[26] Ishibashi K, Imai M. Prospect of a stanniocalcin endocrine/paracrine system in mammals. Am J Physiol Renal Physiol 2002;282:F367.
[27] Ito D, Walker JR, Thompson CS, Moroz I, Lin W, Veselits ML, et al. Characterization of stanniocalcin 2, a novel target of the mammalian unfolded protein response with cytoprotective properties. Mol Cell Biol 2004;24:9456–69.
[28] Nagpal S, Na S, Rathnachalam R. Non-calcemic actions of vitamin D receptor ligands. Endocr Rev 2005;26:662–87.
[29] Blumberg JM, Tzameli I, Astapova I, Lam FS, Flier JS, Hollenberg AN. Complex role of the vitamin D receptor and its ligand in adipogenesis in 3T3-L1 cells. J Biol Chem 2006;281:11205–13.
[30] Garrett JE, Capuano IV, Hammerland LG, Hung BCP, Brown EM, Hebert SC, et al. Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. J Biol Chem 1995;270: 12919–25.
[31] Gensure RC, Gardella TJ, Juppner H. Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. Biochem Biophys Res Commun 2005;328:666–78.
[32] Zemel MB. Mechanisms of dairy modulation of adiposity. J Nutr 2003;133:252S–6S.
[33] Zemel MB. Role of calcium and dairy products in energy partitioning and weight management. Am J Clin Nutr 2004;79:907S–12S.
[34] Kwak JO, Kwak J, Kim HW, Oh KJ, Kim YT, Jung SM, et al. The extracellular calcium sensing receptor is expressed in mouse mesangial cells and modulates cell proliferation. Exp Mol Med 2005;37:457–65.