No effect of dietary calcium on body weight of lean and obese mice and rats

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Zhang, Qinmin, and Michael G. Tordoff. No effect of dietary calcium on body weight of lean and obese mice and rats. Am J Physiol Regul Integr Comp Physiol 286: R669–R677, 2004.—Recent epidemiological and animal studies have led to the hypothesis that low dietary calcium intakes contribute to obesity. Here, we evaluated whether calcium influenced the body weight of normal-weight and obese rodents. All experiments involved female C57BL/6J mice or Sprague-Dawley rats fed normal- or high-energy-density diets (3.8 or 4.7 kcal/g). Calcium intake was manipulated by allowing mice to drink sweetened 30 mM CaCl2 solution or feeding mice and rats diets differing in calcium content (0.2%, 0.6%, or 1.8% Ca2+). Blood samples were taken from rats to confirm that the diets had their intended effects on metabolism. There were no effects of the calcium manipulations on energy intake, body weight, or carcass fat content and no simple relation between calciotropic hormones and body weight. One experiment found a significant decrease in body weight gain of lean and obese rats fed the 1.8% Ca2+ diet, but we suspect that this was due to forced consumption of the unpalatable diet, reducing growth. These studies provide little support for the hypothesis that dietary calcium contributes to the etiology or maintenance of obesity.

1.25-dihydroxyvitamin D3; parathyroid hormone; appetite; palatability; food intake

THERE HAS BEEN CONSIDERABLE recent interest in the hypothesis that body fat stores are modulated by calcium intake (50, 55). Several epidemiological studies have found inverse correlations between calcium intakes and body weight (7, 10, 13, 17, 22–24, 30, 55). There are also reports that body weight is reduced during clinical trials involving calcium supplementation (10, 15, 29, 55). A measure of the enthusiasm for this hypothesis is the number of reviews written about it (28, 35, 48–50, 52).

Experimental support for an inverse relation between calcium intake and body weight has been derived from animal studies. There are several reports of reduced body weight gain in rats fed high-calcium diets relative to controls fed moderate levels of calcium (6, 26, 34, 38, 54). However, interest has been piqued recently by three studies showing that high dietary calcium reduces the obesity produced by feeding high-energy diets. Two studies (33, 55) involved groups of male aP2-agouti mice fed high-sucrose, high-lard diets that differed in the amount and source of calcium (i.e., 0.4% Ca2+ from CaCO3, 1.2% Ca2+ from CaCO3, 1.2% Ca2+ from nonfat dry milk, or 2.4% Ca2+ from nonfat dry milk). In one of these studies (33), food intakes of some groups were yoked to those of mice fed the 0.4% Ca2+ diet. In both experiments, the high-calcium diets significantly reduced weight gain and fat pad mass, increased core body temperature, inhibited adipocyte fatty acid synthase expression, stimulated lipolysis, and reduced fasting plasma insulin and glucose concentrations. The third study (27) involved two groups of male Wistar rats fed diets containing 0.4% or 2.4% Ca2+. The groups had similar food intakes, body temperatures, respiratory quotients, energy expenditures, and fasting serum glucose and insulin concentrations, but the group fed the high-calcium diet had significantly lower weight gain, less carcass fat, greater fecal excretion of lipid, and lower serum triglyceride and 1,25-dihydroxyvitamin D3 [1,25(OH)2D] concentrations.

In the present work, we wanted to conduct a more rigorous investigation of the influence of calcium on dietary obesity. First, whereas the three earlier studies investigated the effect of a high-calcium diet on the development of dietary obesity, we wanted to examine the effect of a low-calcium diet as well. Second, the diets used in earlier studies were high energy density and differed in the amount and source of calcium. We wanted to use well-defined diets that differed solely in calcium content. We also thought it important to include controls fed “normal”–energy-density diets. Third, on the basis of human psychophysical data (40) and studies on calcium solution preference in rodents (1, 38, 41, 43), we suspected that high concentrations of calcium in the diet are unpalatable. Thus we wanted to avoid the possibility that the effect of high dietary calcium to reduce body weight was due simply to the diet’s hedonic properties.

All the experiments we conducted involved comparing the body weight of mice or rats fed diets containing normal (3.8 kcal/g) and “high” (4.6 kcal/g) energy density, with various manipulations of calcium content. In experiment 1, we attempted to increase calcium intake by providing mice with calcium solutions to drink. In experiments 2 and 3, we fed mice and rats diets differing in calcium content. In addition to measures of body weight, blood samples were taken from rats to confirm that the diets had their intended effects on metabolism. Finally, a follow-up experiment examined the relative acceptability of the diets to rats.

METHODS

Animals and Housing

The experiments involved female C57BL/6J mice or Sprague-Dawley rats. The mice (Jackson Laboratory, Bar Harbor, ME) were housed in 28 × 18 × 13-cm plastic shoebox cages with Aspen chip bedding (Pro Chip Aspen, PWI Industries) and stainless steel wire lids. The rats (Charles River Laboratories, Stone Ridge, NY) were housed in 20 × 18 × 25-cm hanging stainless steel cages with mesh floors and front walls. All animals were housed alone and were maintained at 23°C on a 12:12-h light-dark cycle.

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Food and Water

Food and water were available ad libitum throughout all experiments. The food was provided to mice as pellets in a hopper built into the cage lids (2, 42) and to rats as a powder in 120-ml Qorpak glass jars held upright against the front wall of their cage by a stainless steel spring. All food was prepared by Dyets (Bethlehem, PA) and was based on the AIN-76A formulation [no. 200150 (normal-energy-density diet) and no. 213758 (high-energy-density diet), Dyets]. All contained in common (g/kg diet) 200 casein, 50 cellulose, 35 calcium-free salt mix, 10 vitamin mix (no. 300050, Dyets), 2 choline bitartrate, and 3 DL-methionine. For the normal-energy-density diets, carbohydrate was supplied as 150 g of cornstarch and 485 g of sucrose, and fat was supplied as 50 g of corn oil. The gross energy density of this formulation was calculated to be 3.8 kcal/g, with 21%, 67%, and 12% of energy from protein, carbohydrate, and fat, respectively. For the high-energy-density diets, carbohydrate was supplied as 361 g of cornstarch and 104 g of Dyetrose (a proprietary form of cornstarch that allows the diet to be easily pelleted). Fat was supplied as 220 g of corn oil. The gross energy density of this formulation was calculated to be 4.6 kcal/g, with 17%, 42%, and 43% of energy from protein, carbohydrate, and fat, respectively.

The “normal”-calcium diets contained 15 g CaCO₃/kg diet. The low-calcium diets contained 7.5 g (experiment 1) or 5 g (experiments 2–4) of CaCO₃. The high-calcium diets contained 45 g of CaCO₃. The difference in weight from the normal diet due to the CaCO₃ supplements was made up by adding or removing the equivalent weight of cornstarch. Thus the diets used in experiment 1 provided calcium at levels of 0.3% and 0.6% (i.e., 75 and 150 mmol Ca²⁺/kg) and in experiment 2–4 at 0.2%, 0.6%, or 1.8% (i.e., 50, 150, or 450 mmol Ca²⁺/kg). These values were chosen because the 0.6% Ca²⁺ diets are similar in calcium content to the AIN-76A formulation (i.e., 0.52%), the 0.2% Ca²⁺ diets are marginally above levels required to maintain normal growth and bone mineralization [i.e., 0.1–0.17% (5, 8)], and the 1.8% Ca²⁺ diets are close to levels we have found in other strains to reduce body weight (38).

In most circumstances, the mice and rats had deionized water to drink. This was generally provided from inverted 300-ml bottles with neoprene stoppers and stainless steel spouts. However, when fluid intake was measured in mice, fluids were provided in pipettes with graduated scales that allowed volumetric measurements to be made (to the nearest 0.1 ml) (2, 42).

Measurements

Food intake and body weight. Food intakes of rats were measured every other day by weighing each food jar to the nearest 0.1 g and subtracting this weight from the previously collected weight. Spillage was collected from cardboard sheets under the cage and accounted for. Food jars were refilled with fresh food as needed. We attempted to measure food intakes of mice but could not obtain accurate estimates because the pelleted high-energy-density diets tended to crumble and particles spilled into bedding, where it was difficult to collect.

All animals were weighed weekly, to the nearest 0.1 g. Mice were rehoused in clean cages at the time they were weighed.

Blood measures. At the end of experiment 3 (see below), blood was collected from rats to provide a measure of their calcium and energy status. After decapitation, trunical blood was drained into chilled plastic tubes containing 30 μmol of EDTA. The tubes were then centrifuged at 2,000 g for 15 min at 4°C, and aliquots of plasma were frozen at −80°C for future assay. To avoid problems with the chelation of calcium by EDTA, two additional blood samples (30–40 μl each) were collected from the bleeding trunk into heparinized microhematocrit tubes. One was immediately analyzed for ionized calcium using a calcium analyzer (model 634, CIBA-Corning). The other was centrifuged, and 10 μl of plasma were analyzed for total plasma calcium content using a colorimetric method based on the interaction of calcium with o-cresolphthalein complex one (kit no. 587, Sigma Chemical, St. Louis, MO).

Rat parathyroid hormone (PTH) was measured using a 125I-immunoradiometric assay kit (no. 50–2000, Immutopics). This involved a two-antibody assay that recognized intact PTH-(1–84) and the N-terminal region [PTH-(1–34)]. Plasma calcium concentrations were determined using a 125I-IRMA kit (no. 50-5000, Immutopics). This involved a monoclonal antibody that was immobilized onto plastic beads to capture the calcitonin molecules and an affinity-purified polyclonal goat antibody that was radiolabeled with 125I for detection. RIAs were used to measure 1,25(OH)₂D₃ (no. 40-6090, Nichols Institute Diagnostics), corticosterone (no. 07-120102, ICN Pharmaceuticals), and leptin (no. RL-83K, Linco Research). 1,25(OH)₂D₃ was first separated from interfering metabolites by immunoextraction utilizing a specific monoclonal antibody. The hormone was then quantified by RIA utilizing a polyclonal antibody and 125I-labeled 1,25(OH)₂D₃ as a tracer. Plasma insulin concentrations were measured by ELISA (no. INSKR020, Crystal Chem). Briefly, insulin in the plasma sample was simultaneously bound to the mouse anti-insulin monoclonal antibody and the anti-insulin antibody of the guinea pig serum. Horse-radish peroxidase-conjugated anti-guinea pig antibody was further bound to the complex. The conjugate was then detected using o-phenylenediamine substrate solution. Plasma glucose, free fatty acids (NEFAs), triglycerides, and cholesterol were measured using enzyme-based colorimetric methods (no. 994-90902, Wako Pure Chemical Industries; NEFA-C kit, Wako Pure Chemical Industries, and no. F6428 and G7793, Sigma Chemical, respectively). Concentrations of ketone bodies were quantified enzymatically with fluorometric detection according to methods described by Ramirez (32). Because of technical problems, we conducted only single assays of PTH, corticosterone, and insulin. For the other hormones and fuels, the correlation coefficient between duplicate assays was 0.89–0.99.

Body composition. Carcass composition was analyzed according to the procedures described by Ramirez (32). Briefly, each carcass was shaved, and the stomach was emptied to remove its contents. The carcass (including head and stomach) was homogenized in an equal weight of water and then dried to determine carcass water content. Fat content was determined by extracting fat from a few grams of the dried homogenate using repeated washes in petroleum ether. Protein content (lean mass) was calculated from the difference between the defatted sample and the weight of ash remaining after heating at 800°C for 24 h.

Before homogenization, a femur was extracted and analyzed for calcium using methods adapted from those of Donahue et al. (12). The bone was defatted in 2:1 chloroform-menthol, dried at 110°C for 48 h, and weighed, and its volume was determined by displacement. It was then dissolved in 6 N HCl, and aliquots were analyzed for calcium content using the same method used for total calcium in plasma.

Procedures

Experiment 1: C57BL/6J mice given sweetened CaCl₂ solution to drink. Experiment 1 was designed to test the effects of various methods of calcium supplementation on mice fed a high-energy-density, moderately low-calcium diet, which we thought paralleled human consumption (see DISCUSSION). According to a 2 × 4 design, a total of 96 female C57BL/6J mice aged 10–13 wk were fed normal- or high-energy-density diets. Within each diet condition, the animals were randomly assigned to one of four groups that differed in the source of calcium (n = 12/group). Three groups were fed diet containing 0.3% CaCl₂ but differed in what they had to drink. They had continuous access to J) two bottles of deionized water, 2) a bottle of deionized water and a drinking tube of 30 mM CaCl₂ in 10 mM sodium saccharin, or 3) two drinking tubes of 30 mM CaCl₂ in 10 mM sodium saccharin. The concentration of CaCl₂-saccharin mixture was chosen on the basis of strain surveys (1, 43) and pilot work aimed at maximizing CaCl₂ intake. The fourth group was fed diet containing...
0.6% Ca\(^{2+}\) and had water to drink. Thus, relative to the group fed 0.3% Ca\(^{2+}\) with water to drink, there were groups given calcium supplementation by voluntary solution intake, forced solution intake, or forced food consumption. We use “forced” here to imply that the mice had no choice but to consume the calcium to obtain water or energy.

The volume of solution ingested by each mouse was recorded twice a week, and body weights of the animals were monitored weekly for 12 wk.

**Experiment 2:** C57BL/6J mice fed diets differing in energy density and calcium content. The results of experiment 1 taught us that mice would not ingest enough calcium solution to allow this to be a practical method for manipulation of calcium intake (see RESULTS). In experiment 2, we wanted to examine the effect on body weight of a wider range of calcium intakes. Ninety of the mice used in experiment 1 were maintained on the same normal- or high-energy-density diets but were given only water to drink for 12 wk. After this, they were ~36 wk old. The mice in each diet group were then assigned to one of three calcium treatment groups that were matched for previous history (i.e., n = 15/group, with 3 or 4 mice from each of the 4 calcium access conditions used in experiment 1). These new groups were fed diet containing 0.2%, 0.6%, or 1.8% Ca\(^{2+}\). Body weights of the animals were monitored weekly for 6 wk, when the mice were killed by anesthetic overdose (2.1 mg ketamine + 0.3 mg xylazine per 30 g body wt), and their carcass was analyzed for fat content. We planned to conduct body composition analyses on all 90 mice, but the results were clear after analysis of the fat content of half the mice, so data from only these mice (n = 7 or 8/group) are presented.

**Experiment 3:** Sprague-Dawley rats fed diets differing in energy density and calcium content. Experiment 3 was similar to experiment 2, except it involved rats, instead of mice, and an extensive set of physiological measures was collected at the end of the experiment. A total of 81 female Sprague-Dawley rats aged 12 wk were randomly assigned to normal- or high-energy-density diets containing 0.6% Ca\(^{2+}\). They were fed these diets for 10 wk. Some rats are resistant to dietary obesity (9, 21), so, on the basis of body weights of the 22-wk-old rats, nine rats fed the high-energy-density diet that gained the least weight were discarded from the experiment. The remaining 36 rats fed the normal-energy-density diet and 36 rats fed the high-energy-density diet were assigned to subgroups of 12 that were matched for body weight. Each received a diet containing 0.2%, 0.6%, or 1.8% Ca\(^{2+}\) for 52 days. Starting 3 days before the switch in diets, food intakes were measured daily and body weights were measured weekly. After 52 days, at the end of the study, the rats were killed by decapitation to allow collection of blood and measurement of body composition.

**Experiment 4:** choice of calcium-containing diets by Sprague-Dawley rats. A total of 24 naive female Sprague-Dawley rats aged ~17 wk were maintained on the normal-energy-density diet containing 0.6% Ca\(^{2+}\). Each rat was given three 48-h two-cup dietary preference tests. Half of the rats were tested with normal-energy-density diets and the other half with high-energy-density diets. During the test, the animals were given two cups of food, which differed in calcium content: 1) 0.2% vs. 0.6% Ca\(^{2+}\), 2) 0.2% vs. 1.8% Ca\(^{2+}\), or 3) 0.6% vs. 1.8% Ca\(^{2+}\). Food intakes of each diet were measured daily during the test. Each rat was tested three times in counterbalanced order, with 4–7 days between tests, during which they were fed the normal-energy-density diet containing 0.6% Ca\(^{2+}\).

**Statistical Analyses**

Intakes recorded over several days were expressed as daily intakes by dividing by the appropriate number of days. All food intakes were converted to energy intakes by multiplying food weight by its energy density (3 to 4.6 kcal/g for the normal- and high-energy-density diets, respectively). Results were analyzed by ANOVA. For experiments 1–3, the designs had factors of diet energy density (normal or high) and calcium availability (0.3%, 0.3% + voluntary CaCl\(_2\), 0.3% + forced CaCl\(_2\), or 0.6% in experiment 1 and 0.2%, 0.6%, and 1.8% in experiments 2 and 3). An additional within-subject factor was used to incorporate repeated measures into the analysis (e.g., energy intakes). For experiment 4, food intakes and preferences were analyzed using mixed-design ANOVAs with factors of diet energy density and test type (0.2% vs. 0.6%, 0.2% vs. 1.8%, and 0.6% vs. 1.8%). The critical level of statistical significance for all tests was P < 0.05. Values are means ± SE.

**RESULTS**

**Experiment 1:** C57BL/6J Mice Given Sweetened CaCl\(_2\) Solution to Drink

Neither voluntary nor forced consumption of sweetened 30 mM CaCl\(_2\) solution was sufficient to cause a marked influence on calcium intake. Average daily intakes of CaCl\(_2\) solution by mice given a choice of water and sweetened CaCl\(_2\) were 2.7 ± 0.5 ml/day (3.3 ± 0.6 mg Ca\(^{2+}\)/day) for those fed the normal-energy-density diet and 3.5 ± 0.4 ml/day (3.9 ± 0.5 mg Ca\(^{2+}\)/day) for those fed the high-energy-density diet. When no water was available so CaCl\(_2\) intake was forced, the mice drank significantly more sweetened CaCl\(_2\) solution. Intakes were 4.3 ± 0.8 ml/day (5.2 ± 0.9 mg Ca\(^{2+}\)/day) for those fed the normal-energy-density diet and 3.7 ± 0.5 ml/day (4.4 ± 0.6 mg Ca\(^{2+}\)/day) for those fed the high-energy-density diet. Under conditions identical to those used here, C57BL/6J mice consume ~4 g food/day (2, 3), which would provide ~12 mg Ca\(^{2+}\)/day if the mice were fed the 0.3% Ca\(^{2+}\) diets or ~24 mg Ca\(^{2+}\)/day if they were fed the 0.6% Ca\(^{2+}\) diets used here. Thus all the mice fed the 0.3% Ca\(^{2+}\) diet consumed less calcium than mice fed the 0.6% Ca\(^{2+}\) diet, even though some of them were forced to drink sweetened CaCl\(_2\) solution.

At the end of the 12-wk test, the mice fed the high-energy-density diets were significantly heavier and fatter than those fed the normal-energy-density diets [F(1,88) = 15.5, P < 0.0001]. However, the manipulations of calcium intake had no significant effect on body weight [F(3,88) = 1.19, P = 0.32; Table 1].

**Experiment 2:** C57BL/6J Mice Fed Diets Differing in Energy Density and Calcium Content

The mice fed the high-energy-density diets were significantly heavier than those fed the normal-energy-density diets before the diet calcium manipulation, and this difference per-
sisted over the 12-wk test \[F(1,67) = 309.1, \ P < 0.001; \text{Table 2}\]. Diet calcium concentration had no effect on final body weight or percent fat content [weight: \(F(2,60) = 0.03, \ P = 0.97\); percent fat: \(F(2,35) = 0.11, \ P = 0.90; \text{Table 2}\).

**Experiment 3: Sprague-Dawley Rats Fed Diets Differing in Energy Density and Calcium Content**

Body weight, body weight gain, and food intake. The high-energy-density diet had its intended effect of increasing body weight. Immediately before the rats first received diets differing in calcium content, those fed the high-energy-density diet for the previous 10 wk were significantly heavier than those fed the normal-energy-density diet \[F(1,66) = 29.3, \ P < 0.0001\]. In confirmation of the assignment of rats to matched groups, there were no differences in body weight among the three subgroups fed diets of the same energy density [main effect of calcium: \(F(2,66) = 0.00, \ P = 0.99\); energy density \(\times\) calcium interaction: \(F(2,66) = 0.04, \ P = 0.96; \) Fig. 1, Table 3].

At every time it was measured during the 52 days that diet calcium content was manipulated, body weight remained significantly increased by the high-energy-density diet but was unaffected by diet calcium content (Fig. 1). There was a tendency for rats fed the 1.8% Ca\(^{2+}\) diet, particularly the normal-energy-density version, to weigh less than the other groups, but this was not significant [at week 8, main effect of calcium: \(F(2,66) = 1.81, \ P = 0.17\); energy density \(\times\) calcium interaction: \(F(2,66) = 0.05, \ P = 0.96; \) Table 3]. However, there were differences in body weight gain (i.e., the difference in weight between the end and the beginning of the 52-day test). Rats fed the high-energy-density diets gained significantly more weight than those fed the normal-energy-density diets \[F(1,66) = 14.9, \ P = 0.0003\]. Moreover, rats fed the 1.8% Ca\(^{2+}\) diets gained significantly less weight than those fed the 0.2% or 0.6% Ca\(^{2+}\) diets [main effect: \(F(2,66) = 7.82, \ P = 0.0009\)]. There was a tendency for the difference to be larger for rats fed the normal-energy-density diet relative to the high-density-diet comparison, but the interaction was not significant [energy density \(\times\) calcium interaction: \(F(2,66) = 0.023, \ P = 0.79; \) Table 3]. Post hoc comparisons (which are arguably not appropriate when the interaction term is nonsignificant) found a significant difference in weight gain among the three groups fed the normal-energy-density diet \[F(2,33) = 7.11, \ P = 0.0027\] but no significant difference in weight gain among the three groups fed the high-energy-density diet \[F(2,33) = 2.92, \ P = 0.068\].

Over the entire test period, rats fed the high-energy-density diets consumed more energy than rats fed the normal-energy-density diets \[F(1,66) = 12.4, \ P = 0.0008\]. Diet calcium had no effect on energy intake \[F(2,66) = 1.08, \ P = 0.34; \] energy density \(\times\) calcium interaction: \(F(2,66) = 0.75, \ P = 0.47; \) Table 3].

**Body composition.** Rats fed the high-energy-density diets had significantly less water [percentage of carcass: \(F(1,66) = 35.0, \ P < 0.0001\); total: \(F(1,66) = 13.4, \ P = 0.0005\)], significantly more fat [percentage of carcass: \(F(1,66) = 38.7, \ P < 0.0001\); total: \(F(1,66) = 36.5, \ P < 0.0001\)], and significantly more total carcass protein [\(F(1,66) = 7.30, \ P = 0.0088\)] than rats fed the normal-energy-density diets. Diet calcium content had no effect on any component of body composition, and there were no interactions between diet calcium and diet energy density. However, post hoc analyses of each diet condition showed that the group fed the normal-energy-density diet containing 1.8% Ca\(^{2+}\) had significantly less carcass protein than the groups fed the normal-energy-density diet containing 0.2% or 0.6% Ca\(^{2+}\) [\(F(2,33) = 3.29, \ P = 0.049\)]. This was not significant for the same comparison of carcass fat content \[F(2,33) = 2.48, \ P = 0.10\] or for the similar comparison of groups fed the high-energy-density diets (Table 3).

Table 2. Body weights of female C57BL/6J mice fed diets differing in energy and calcium content for 12 wk

<table>
<thead>
<tr>
<th>Measure</th>
<th>Normal-Energy-Density Diets</th>
<th>High-Energy-Density Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2% Ca(^{2+})</td>
<td>0.6% Ca(^{2+})</td>
</tr>
<tr>
<td></td>
<td>0.2% Ca(^{2+})</td>
<td>0.6% Ca(^{2+})</td>
</tr>
<tr>
<td>Weight before Ca(^{2+}) intervention, g</td>
<td>27±0.6</td>
<td>27±0.5</td>
</tr>
<tr>
<td>Final weight, g</td>
<td>25±0.6</td>
<td>25±0.6</td>
</tr>
<tr>
<td>Carcass fat content, % total body wt</td>
<td>15±3</td>
<td>15±1</td>
</tr>
</tbody>
</table>

Values are means ± SE. 0.2%, 0.6%, and 1.8% Ca\(^{2+}\) refer to groups of 15 mice fed these concentrations of calcium in the diet. For all 3 measures, values were significantly higher in mice fed high-energy-density diets than in those fed normal-energy-density diets. No differences were observed among calcium groups.

Fig. 1. Body weight and energy intake of female Sprague-Dawley rats fed normal- or high-energy-density diets with 0.2%, 0.6%, or 1.8% Ca\(^{2+}\). Rats fed high-energy-density diets (4.6 kcal/g) were significantly heavier at all times and over the entire test consumed significantly more energy than rats fed the normal-energy-density diets (3.8 kcal/g). There were no significant effects in either measure related to diet calcium content.
### Table 3. Body weight, body composition, energy intake, and femur calcium content of Sprague-Dawley rats fed diets differing in energy density and calcium content for 10 wk

<table>
<thead>
<tr>
<th></th>
<th>Normal-Energy-Density Diets</th>
<th>High-Energy-Density Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2% Ca²⁺</td>
<td>0.6% Ca²⁺</td>
</tr>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>380±9</td>
<td>379±9</td>
</tr>
<tr>
<td>Final Change</td>
<td>436±13</td>
<td>436±11</td>
</tr>
<tr>
<td>Food intake, kcal/day</td>
<td>67±2</td>
<td>68±2</td>
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<tr>
<td><strong>Body composition</strong></td>
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<td></td>
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<tr>
<td>Water, g</td>
<td>205±3</td>
<td>197±7</td>
</tr>
<tr>
<td>Protein, g</td>
<td>73±3</td>
<td>70±3</td>
</tr>
<tr>
<td>Fat, g</td>
<td>153±11</td>
<td>164±11</td>
</tr>
<tr>
<td>Fat content, %body wt</td>
<td>34±2</td>
<td>37±2</td>
</tr>
<tr>
<td><strong>Femur</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, mg</td>
<td>571±13</td>
<td>546±12</td>
</tr>
<tr>
<td>Density, g/ml</td>
<td>1.03±0.02</td>
<td>1.11±0.02</td>
</tr>
<tr>
<td>Ca²⁺ per bone, mg</td>
<td>134±4</td>
<td>129±3</td>
</tr>
<tr>
<td>Ca²⁺ density, mg/g</td>
<td>234±3</td>
<td>257±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. 0.2%, 0.6%, and 1.8% Ca²⁺ refer to groups fed these concentrations of calcium in the diet (n = 12/group). Start, before Ca²⁺ intervention but after 10 wk of normal- or high-energy-density diet containing 0.6% Ca²⁺. Femur weight represents weight of dry, defatted femur. *P < 0.05 relative to the other 2 groups fed diets of the same energy density, by planned comparisons. There were significant differences between high- and normal-energy-density diet groups in all measures except femur weight and femur calcium per bone.

**Femur calcium.** The manipulations of diet energy density and calcium content had no effect on femur weight or calcium content. However, rats fed the high-energy-density diets had femurs with significantly smaller volumes [F(1,66) = 10.6, P = 0.0018; data not shown], higher density [F(1,66) = 10.3, P = 0.0021], and higher calcium density [i.e., calcium per gram of bone; F(1,66) = 13.9, P = 0.0004; Table 3].

**Plasma hormones and fuels.** Rats fed high-energy-density diets had significantly higher plasma concentrations of calcitonin, insulin, leptin, glucose, and triglycerides and significantly lower plasma concentrations of 1,25(OH)₂D and PTH than rats fed normal-energy-density diets (P < 0.014 for each). Diet energy density had no significant effect on ionized calcium, total calcium, corticosterone, free fatty acids, or ketones (Table 4).

Diet calcium content had significant effects on blood ionized calcium, plasma total calcium, 1,25(OH)₂D, and PTH (P < 0.0001 for each). Rats fed the 1.8% Ca²⁺ diets had significantly higher concentrations of ionized and total calcium and significantly lower concentrations of PTH than rats fed the 0.2% or 0.6% Ca²⁺ diets. Rats fed the 0.2% Ca²⁺ diets had significantly higher concentrations of 1,25(OH)₂D than those fed the 0.6% or 1.8% Ca²⁺ diets. There were no significant effects of diet calcium on plasma concentrations of calcitonin, corticosterone, insulin, leptin, or any of the metabolic fuels.

For two of the measures, 1,25(OH)₂D and PTH, there was an interaction between diet energy density and calcium content [F(2,66) = 9.23, P = 0.0003, and F(2,66) = 3.65, P = 0.031, respectively]. This was due to rats fed the low-calcium, high-energy-density diets having lower hormone concentrations than the equivalent groups fed the normal-energy-density diets. In other words, rats fed the high-energy-density diets appeared to have a blunted 1,25(OH)₂D and PTH response to moderate or low levels of calcium.

### Table 4. Calcium, hormones, and metabolic fuel concentrations in blood of Sprague-Dawley rats fed diets differing in energy density and calcium content for 10 wk

<table>
<thead>
<tr>
<th></th>
<th>Normal-Energy-Density Diets</th>
<th></th>
<th></th>
<th>High-Energy-Density Diets</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2% Ca²⁺</td>
<td>0.6% Ca²⁺</td>
<td>1.8% Ca²⁺</td>
<td>0.2% Ca²⁺</td>
<td>0.6% Ca²⁺</td>
<td>1.8% Ca²⁺</td>
</tr>
<tr>
<td>Blood ionized Ca²⁺, mmol/l</td>
<td>1.23±0.02*</td>
<td>1.26±0.05*</td>
<td>1.42±0.03†</td>
<td>1.23±0.04*</td>
<td>1.31±0.02†</td>
<td>1.35±0.02‡</td>
</tr>
<tr>
<td>Plasma total Ca²⁺, mmol/l</td>
<td>2.84±0.04*</td>
<td>2.88±0.04*</td>
<td>3.02±0.04‡</td>
<td>2.81±0.03*</td>
<td>2.86±0.07*</td>
<td>3.05±0.05‡</td>
</tr>
<tr>
<td>1,25(OH)₂D, pg/ml</td>
<td>57.8±9.08</td>
<td>23.7±7.32</td>
<td>2.5±0.5*</td>
<td>19.4±3.6†</td>
<td>5.9±1.4†</td>
<td>7.6±2.3‡†</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>70.6±9.2†</td>
<td>78.1±17.1</td>
<td>10.2±2.0*</td>
<td>61.3±6.0†</td>
<td>34.1±5.5</td>
<td>10.2±1.6*</td>
</tr>
<tr>
<td>Calcitonin, pg/ml</td>
<td>479±81*</td>
<td>516±70*</td>
<td>489±72*</td>
<td>1.008±15†</td>
<td>674±89*</td>
<td>1.015±137†</td>
</tr>
<tr>
<td>Insulin, pg/ml</td>
<td>4.8±0.7*</td>
<td>5.8±0.8†</td>
<td>3.7±0.5*</td>
<td>9.2±1.2†</td>
<td>9.6±1.6†</td>
<td>7.7±0.8†</td>
</tr>
<tr>
<td>Leptin, pg/ml</td>
<td>23.9±4.0*</td>
<td>27.8±4.1*</td>
<td>16.2±2.6*</td>
<td>56.8±6.2†</td>
<td>51.3±7.9†</td>
<td>54.7±6.6†</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>261±50*</td>
<td>388±52*</td>
<td>260±48*</td>
<td>293±46‡</td>
<td>339±52*</td>
<td>285±53*</td>
</tr>
<tr>
<td>Glucose, mg/ml</td>
<td>1.34±0.08*</td>
<td>1.50±0.08†</td>
<td>1.47±0.12‡</td>
<td>1.66±0.08*</td>
<td>1.71±0.13†</td>
<td>1.64±0.09†</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>1.16±0.12†</td>
<td>1.56±0.17†</td>
<td>1.10±0.19*</td>
<td>1.39±0.19†</td>
<td>1.56±0.32†</td>
<td>1.74±0.23†</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.58±0.06*</td>
<td>0.79±0.10†</td>
<td>0.57±0.07*</td>
<td>0.86±0.11†</td>
<td>0.82±0.09†</td>
<td>0.86±0.10†</td>
</tr>
<tr>
<td>Ketones, mmol/l</td>
<td>0.11±0.01*</td>
<td>0.13±0.01†</td>
<td>0.15±0.02*</td>
<td>0.12±0.00†</td>
<td>0.13±0.01†</td>
<td>0.14±0.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE. 0.2%, 0.6%, and 1.8% Ca²⁺ refer to groups fed these concentrations of calcium in the diet (n = 12/group). Values in the same row with the same symbol do not differ significantly from each other. 1.25(OH)₂D, 1.25-hydroxyvitamin D₃; PTH, parathyroid; FFA, free fatty acid.
Fig. 2. Food intake of female Sprague-Dawley rats given 48-h choice tests between pairs of diets differing in calcium content. Percentages above bars are preference scores for the diet containing the lesser amount of calcium in each pair. \( P < 0.05 \) relative to intake of other diet available; \( P < 0.05 \) relative to intake of similar normal-energy-density diet in the same test.

Experiment 4: Choice of Calcium-Containing Diets by Sprague-Dawley Rats

When allowed to choose between pairs of diets with different calcium levels, rats in all groups strongly preferred the food containing less calcium (Fig. 2). In all tests, rats ate significantly more of the lower-Ca\(^{2+}\) diet than the higher-Ca\(^{2+}\) alternative [\( F(1,22) = 153.4, P < 0.00001 \)]. This was reflected in preference scores (Fig. 1). There were no differences in the total amount of food ingested among any of the tests.

In tests involving the diet containing 1.8% Ca\(^{2+}\), avoidance of the high-calcium alternative was significantly stronger when the calcium was presented in the normal-energy-density, rather than the high-energy-density, diet [\( F(1,22) = 7.25, P = 0.013 \)]. This effect was also present in analyses of preference scores: Rats given a choice of calcium mixed in normal-energy-density diets avoided the high-calcium alternative more than did those given the same amounts of calcium mixed in the high-energy-density diets [\( F(2,44) = 20.8, P < 0.00001 \); Fig. 2].

**DISCUSSION**

The present results provide little support for the hypothesis that murine dietary obesity is influenced by calcium consumption. Experiments 2 and 3 involved parametric manipulations of diet energy density and calcium content in mice and rats. In both experiments, neither body weight nor body fat content was influenced by a wide range of calcium concentrations, and neither was influenced by the interaction of diet calcium content with diet energy density. Some caution is required in interpretation of the results with rats, because those fed 1.8% Ca\(^{2+}\) diets gained significantly less weight than those fed 0.2% or 0.6% Ca\(^{2+}\), which is consistent with an inverse relation between calcium intake and obesity. However, the effect on body weight gain was 1) not present in absolute body weights, 2) not present in mice, 3) not “dose” related, 4) if anything, greater in lean than in obese rats, and 5) accompanied by lower carcass protein weights in the rats fed normal-energy-density diets. We interpret these findings to indicate that the high-calcium diets reduced body weight gain by retarding growth and, thus, body size, not by acting specifically on fat stores.

In contrast to our results, some investigators have reported that feeding mice or rats high-calcium diets significantly reduced adipose tissue weights (27, 33, 55). However, in these earlier studies, the effects of obesity and body size on adipose tissue weight are confounded. The designs did not include animals fed normal-energy-density diets, so it is unclear whether differences in weight gain were due to growth or dietary obesity. All three studies involved young male mice or rats that typically grow rapidly. Shi et al. (33) and Zemel et al. (55) did not provide an independent measure of body size (e.g., lean tissue weight or body length). Papakonstantinou et al. (27) found that the carcasses of control and calcium-supplemented rats differed in weight by 32 g, of which 21 g were due to a difference in protein content (neither difference was significant). This was similar to the results of our experiment using female Sprague-Dawley rats, which were growing moderately: The differences in weight gain related to dietary calcium were reflected by a significant difference in carcass protein content. Finally, in our experiment involving 36-wk-old female mice, there was no change in body weights of controls, suggesting that they were no longer growing, and no effect of calcium on body weight gain or carcass fat content. In summary, we believe that the results of all five experiments are consistent with an action of high dietary concentrations of calcium to reduce growth and that calcium-supplemented animals have less body fat simply because they are smaller.

Of course, other aspects of methodology could also contribute to the different outcomes between our experiments and earlier studies. There were differences in diet calcium content (0.2%, 0.6%, and 1.8% Ca\(^{2+}\) vs. 0.4%, 1.2%, and 2.4%) and source (CaCO\(_3\) vs. CaCO\(_3\) and nonfat dry milk). There were also differences in the animals’ gender (female vs. male) and strain (C57BL/6J vs. agouti and Sprague-Dawley vs. Wistar). The use of agouti mice in earlier work may be particularly critical, because these mice have a genetic lesion influencing calcium metabolism (16, 19, 47) and so may be unusually sensitive to manipulations influencing calcium status. One of the earlier studies also involved food restriction (33), which may improve test sensitivity, because the effects of calcium on body weight of humans may be clearer when energy intakes are low (22). Another difference was that our studies involved testing animals that were already obese, whereas the earlier studies involved testing animals during the development of obesity.

Our finding that the AIN-76A-based 1.2% Ca\(^{2+}\) diet reduced the body weight gain of Sprague-Dawley rats is consistent with earlier work showing that body weights of Wistar-Kyoto and spontaneously hypertensive rats were reduced by similar diets containing the same or greater concentrations of calcium (26, 34, 38). The finding that calcium influenced body weight gain significantly in rats fed normal- but not high-energy-density diet appears contrary to a recent abstract involving aP2-agouti mice (54) but consistent with another involving Zucker rats (6). The finding that weight gain was retarded significantly in rats fed normal- but not high-energy-density diets argues against an effect of calcium on fat stores, because the largest effects of calcium were seen in the animals with the least body fat. However, it is unclear what other mechanisms might be involved, because none of the physiological measures we collected varied in parallel with body weight gain; the only
measure that did vary consistently with body weight gain was diet palatability (see below).

We measured several blood factors in rats fed diets differing in energy density and calcium content. Rats fed the high-energy-density diet had the expected increases in plasma insulin, leptin, glucose, and triglyceride concentrations, but none of these were influenced by diet calcium content. Rats fed the 1.8% Ca\(^{2+}\) diet had the expected increases in ionized and total calcium concentrations and decrease in PTH concentrations. Rats fed the 0.2% Ca\(^{2+}\) diets had the expected increases in 1,25(OH)\(_2\)D concentrations. In a few cases, diet energy density influenced measures of calcium metabolism. Interestingly, femur calcium density was significantly higher in animals fed high-energy-density diets than in those fed normal-energy-density diets, although there was no difference in the total amount of calcium in the bone. The rats fed high-energy density diets had femurs with smaller volumes but higher density. Animals fed the high-energy-density diet also had significantly elevated calcitonin concentrations and lower PTH and 1,25(OH)\(_2\)D concentrations. The effects with PTH and 1,25(OH)\(_2\)D were evident for the 0.2% and/or 0.6% Ca\(^{2+}\) concentrations only, probably because the 1.8% Ca\(^{2+}\) diets reduced levels of these hormones to almost zero.

One impetus for conducting this study was to evaluate whether the “bad” taste of calcium was responsible for the reduced body weight of animals forced to eat it. There was little doubt that high concentrations of calcium were disliked. We were unable to coax mice to drink enough 30 mM CaCl\(_2\) solution to increase their calcium intake much, even though we added saccharin to the calcium solution. Moreover, we found that rats strongly avoided high-calcium diets when given a choice to do so. One unexpected finding was that rats avoided calcium less when it was presented in the high- rather than in the normal-energy-density diets. We suspect that this was because the high-energy-density diets contained more fat, and calcium forms insoluble complexes with fat that may prevent it from being tasted.

It is reasonable to expect that daily food intake would be reduced if food is unpalatable, but, in agreement with several other studies (26, 27, 33, 55), we found no difference in daily food intake related to dietary calcium content. Perhaps the requirements for energy or protein override considerations of taste. This is consistent with experiments in which distasteful foods are given deliberately (20, 31); although intakes drop initially, they rebound to normal levels within a few days. It is possible that food taste alters meal patterns, stress hormones, or fuel oxidation in a manner that influences lipogenesis, although we found no differences related to diet in plasma corticosterone concentrations here. It thus remains unclear whether the bad taste of calcium is a cause of the reduced weight gain produced by calcium supplementation.

Other hypotheses linking calcium intake to body weight also fail to explain the results. One involves circulating PTH and 1,25(OH)\(_2\)D concentrations: High concentrations of these hormones can induce high intracellular calcium concentrations in adipose tissue, and these, in turn, stimulate lipogenesis (i.e., obesity) (51, 53, 55). However, rats fed vitamin D-deficient diets have normal body weights, unless they are also calcium deficient (44), and chronic infusions of PTH or 1,25(OH)\(_2\)D do not increase body weight (36). Moreover, here and in the study by Papakonstantinou et al. (27), there was no clear relation between circulating concentrations of 1,25(OH)\(_2\)D (or PTH in this study) and body weight of rats given diets differing in calcium content (27). Taken together, we believe that these data effectively refute the hypothesis that 1,25(OH)\(_2\)D mediates dietary obesity, at least in rats.

An alternative hypothesis was provided by Papakonstantinou et al. (27). These investigators found that fecal fat loss was higher in rats fed high-calcium diets than in those fed moderate-calcium diets, leading to the possibility that the reduced weight gain of rats fed high-calcium diets was due to the sequestration and subsequent loss of fat. Although the sequestration of fat by calcium has been clearly demonstrated (11, 46), it most likely can account for very little weight loss, particularly in humans, where the ratio of calcium to fat intake is very low (11, 28, 48). Unfortunately, we did not measure fecal fat content in our studies. However, our physiological measures do not support the possibility that rats fed high-fat (i.e., high-energy-density) diets absorbed markedly less calcium than did those fed low-fat (normal-energy-density) diets. In particular, diet energy density had no effect on femur calcium content. In fact, the opposite may be true because concentrations of 1,25(OH)\(_2\)D and PTH, which are proxy measures of calcium deficiency, were lower in high-fat-fed rats than in low-fat-fed rats.

 Perspectives

The experiments reported here do not support the hypothesis that there is an inverse relation between calcium intake and obesity in rodents. Our work raises questions about the appropriate animal model for studying the effects of calcium on body weight of humans. First, we suggest that future studies use animals that are fully grown to avoid problems with confounding growth and obesity. We note that high-calcium diets can reduce body weight gain of growing animals (27, 33, 55; experiment 3), but we suspect that this is due to reduced body size, rather than reduced obesity. Second, we believe that greater consideration must be paid to the correspondence between levels of calcium in the animal and human diet. Diets containing high concentrations of calcium are strongly avoided by rodents, and we suspect they have deleterious physiological effects. Rats fed the 1.8% Ca\(^{2+}\) diet used here consumed ~9 mmol Ca\(^{2+}\)/day, but intusions of as little as 3.75 mmol CaCl\(_2\)/day cause malaise [assessed by conditioned taste aversion (39)]. Humans, too, dislike high concentrations of calcium (37, 40), but unlike most laboratory animals, they do not have to choose between eating calcium and starvation. It may be more appropriate to compare rodents fed low-calcium diets with those fed moderate-calcium diets, because these more closely model the human condition of marginal calcium deficiency and moderate calcium supplementation. It is noteworthy in this regard that no animal study has found that low dietary calcium increases body weight or exacerbates dietary obesity.

Our results with rodents have led us to critically evaluate the evidence that calcium reduces obesity in humans. Most of the calcium supplementation studies have been retrospective analyses, and these are subject to reporting bias. Epidemiological studies are difficult to interpret, because calcium intake covaries with the intake of several other nutrients (4, 14, 18, 25), some of which are just as likely as calcium to influence obesity. In rats, macronutrient selection is influenced by calcium status.
(45). It may be that humans provided with calcium alter their food selection, and this contributes to changes in body weight. In summary, we believe that the extant data do not support a simple relation between dietary calcium and obesity in rodents, and the issue remains an open question for humans.

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