Dietary Restriction of Energy and Calcium Alters Bone Turnover and Density in Younger and Older Female Rats1,2,3

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ABSTRACT To determine the influence of weight loss with or without adequate calcium intake on bone turnover and density, we examined the influence of dietary restriction of calcium or energy on body weight (BW), bone mineral density (BMD) and bone turnover in both younger (3 mo) and older (10 mo) female rats (n = 66). Diets were designed to allow feeding at two levels of calcium intake (normal = 78 mg/d and low = 15 mg/d) and two levels of energy intake (normal and 40% restriction) while keeping the intake of protein, fat, fiber, vitamins and other minerals equal between groups. Thus rats received either a control diet (CNTL), a diet restricted in calcium, energy or both for 9 wk. Energy restriction reduced BW 5±21% (P < 0.01) and elevated bone formation 10–20% (P < 0.05) in both age groups. Bone resorption was 20–40% above CNTL values (P < 0.05), in rats fed all three restricted diets. In younger rats, BMD increased over time in all groups (P < 0.05), but final BMD was lower in calcium restricted groups compared with CNTL (P < 0.01). In older rats, CNTL had a significantly greater final BMD (P < 0.05) than diet-restricted groups. These data indicate that, in both younger and older rats, dietary restriction of calcium or energy results in an elevated rate of bone turnover. BMD is compromised by calcium restriction in both younger and older rats, whereas only older rats were negatively influenced by dietary energy restriction. Thus the present study indicates a detrimental effect of low-energy diets, as well as inadequate calcium intake, on bone density in mature rats. J. Nutr. 128: 640–645, 1998.

KEY WORDS: bone density · bone turnover · calcium · energy restriction · rats

Body weight has been shown to be a consistent predictor of bone mineral density (BMD)5 (Holbrook and Barrett-Connor 1993), and weight loss has been shown to result in decreased BMD in both humans (Compston et al. 1992, Hyldestrup et al. 1993, Jensen et al. 1994, Svensden et al. 1993) and rats (Roudubush et al. 1993, Wronek et al. 1987). Several mechanisms have been proposed for the loss of BMD after body weight reduction, including reduced mechanical loading, altered hormone levels and dietary factors such as reduced calcium and energy intake. It is unclear, however, whether the altered rate of bone turnover (Hyldestrup et al. 1993, Svensden et al. 1993) and reduced BMD observed after weight loss are influenced equally by inadequate dietary calcium and reduced energy intake (Lee et al. 1986, Ramsdale and Bassey 1994).

Previous studies in rats examining the influence of calcium or energy restriction on bone turnover and density (Lee et al. 1986 and 1993, Ndiaye et al. 1992 and 1995, Peterson et al. 1995, Shires et al. 1980, Wright and McMillan 1994) have differed widely in the method and degree of dietary restriction. For example, previous investigators have restricted energy intake for 4 wk (Ndiaye et al. 1995) to 4 mo (Lee et al. 1993), restricted calcium intake for 4 d (Egger et al. 1994) to 8 wk (Peterson et al. 1995) and imposed complete food deprivation for 12 h (Wright and McMillan 1994) to 72 h (Ndiaye et al. 1992). Additionally, these studies have utilized rats of varying age from weaning (Ndiaye et al. 1995) to 16 mo (Lee et al. 1986). It is still unclear, however, whether reduced energy intake and weight loss influence the effect of low or adequate calcium intake on bone turnover and density and whether this effect is dependent on age. Therefore this study examined the relationship between calcium and energy intake on bone turnover and density using both a younger and older female rat model.

MATERIALS AND METHODS

Animals. Two age groups of female Sprague-Dawley rats (Taconic Farms, MD) were examined in this study; “younger” (3 mo old, n = 22) and “older” (10 mo old, n = 44). Rats were housed in hanging wire-bottom cages and maintained on a 12 h light/dark cycle with a constant room temperature. Rats were weighed using an XT top-loading balance scale (Fisher Scientific, Pittsburgh, PA), matched for body weight and assigned to one of four diet groups, representing two levels of energy intake and two levels of mineral intake. All procedures were approved by the Rutgers University Institutional Review Board for the Use and Care of Animals.

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3 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.
4 Abbreviations used: BMD, bone mineral density; CR, calcium restriction; ER, energy restriction; CER, calcium and energy restriction; CNTL, control; DXA, dual energy x-ray absorptiometry; [3H]TC, tritiated tetracycline.

TABLE 1
Composition and calcium and energy content of control and restricted diets1,2

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CNTL</th>
<th>CR</th>
<th>ER</th>
<th>CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (g diet)</td>
<td>16.3</td>
<td>16.6</td>
<td>16.1</td>
<td>16.5</td>
</tr>
<tr>
<td>Calcium (Total weight)</td>
<td>698.1</td>
<td>685.1</td>
<td>488.1</td>
<td>475.1</td>
</tr>
<tr>
<td>Salt mix S10001A3</td>
<td>17.5</td>
<td>17.5</td>
<td>17.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>6.5</td>
<td>2.5</td>
<td>6.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total weight</td>
<td>998.1</td>
<td>985.1</td>
<td>608.1</td>
<td>595.1</td>
</tr>
<tr>
<td>Calcium (mg/g diet)</td>
<td>5.2</td>
<td>1.0</td>
<td>8.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Energy (kJ/g diet)</td>
<td>16.3</td>
<td>16.6</td>
<td>16.1</td>
<td>16.5</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>68</td>
<td>68</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Protein</td>
<td>21</td>
<td>21</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Fat</td>
<td>11</td>
<td>11</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

1 Preparing by Research Diets, New Brunswick, NJ. CNTL, control diet; CR, calcium restricted; ER, energy restricted; CER, calcium and energy restricted.
2 The salt mixture (Research Diets) composition was as follows: 0.5 g Mg, 3.6 g K, 0.33 g S, 1.0 g Na, 1.6 g Cl, 2.0 mg Cr, 6.0 mg Cu, 0.2 mg I, 45.0 mg Fe, 59.0 mg Mn, 0.16 mg Se and 29.0 mg Zn.
3 The vitamin mixture (Research Diets) composition was as follows: 2.2 mg retinol palmitate, 0.025 mg cholecalciferol, 50 mg dl-α-tocopheryl acetate, 0.5 mg menadione, 0.2 mg biotin, 10 μg cyanocobalamin, 2 mg folic acid, 30 mg nicotinic acid, 16 mg calcium pantothenate, 7 mg pyridoxine, 6 mg riboflavin and 6 mg thiamin.

Diet. During the 6-wk labeling phase of this experiment, rats had free access to tap water and a customized control diet (CNTL) based on AIN-76 (Reeves et al. 1993), and containing 0.525% calcium and 0.35% phosphorous (Ca:P ratio = 1.5). This level of calcium and phosphorous intake has been shown to be sufficient for normal growth and bone mineralization (Persson et al. 1993). Diets (Research Diets, New Brunswick, NJ) were formulated to provide two levels of energy intake (normal and 40% restriction) and two levels of calcium intake (normal = 78 mg/d and low = 15 mg/d; see Table 1). Daily intakes of protein, fat, fiber, vitamins and other minerals were the same in each diet group. Diet-restricted rats were pair-fed to the mean daily intake of control rats in the same age group. Younger rats consumed ~8 and 13 g/d in the energy-restricted (131 kJ/d) and energy-adequate (214 kJ/d) groups, respectively. Older rats consumed ~11 and 18 g/d in the energy-restricted (180 kJ/d) and energy-adequate (296 kJ/d) groups, respectively. Energy restriction was accomplished by reducing the carbohydrate content (sucrose and corn starch) and feeding a reduced quantity of both energy-restricted diets. In calcium-restricted diets, phosphorous was reduced proportionally to maintain the Ca:P ratio and minimize the possible occurrence of kidney calcinosis, which is common in aged female rats (Reeves et al. 1993). Thus rats in the diet-treatment groups were either restricted in calcium only (CR), energy only (ER) or calcium plus energy (CER).

Tritiated tetracycline ([3H]TC) labeling. After 1 wk of acclimation to our animal care facility, rats were labeled chronically with tritiated tetracycline ([3H]TC) by the method of Muhlbaier and Fleisch (1990). Briefly, rats were injected subcutaneously with a solution containing 370 MBq/L of [3H]TC (New England Nuclear, Boston, MA) dissolved in 0.15 mol/L NaCl. Injections were performed twice weekly for 6 wk using increasing amounts of [3H]TC solution (i.e., 50, 100, 150, 200, 250 and 250 μL per rat). Each rat received a total dose of 740 kBq of tritium. A 10-d equilibration period was allowed between the final injection and dietary intervention to allow the [3H]TC label to reach a steady state before baseline measurements.

[3H]TC determination. [3H]TC content of bones and soft tissues was determined for rats in both age groups. After the 10-d equilibration period, rats were killed by decapitation after CO₂ exposure. Long bones, vertebrae and soft tissues (liver, heart, kidney, hindlimb muscle, lung and fat pad) were dissected, cleaned and weighed. [3H]TC was extracted by HCl hydrolysis at 4°C for 3 d by the method of Jackman et al. (1973). Tissues were washed daily with distilled water, and new acid was added. Washings and acid were pooled. Tritium content was measured in 0.5-μL aliquots added to 3 mL of liquid scintillation cocktail (Ready Safe, Beckman Instruments, Fullerton, CA) by liquid scintillation counting (Tricorn 1900-CA, Packard Instruments, Meriden CT).

Urinary excretion of [3H]TC was measured weekly to estimate bone resorption. Rats were housed individually, once per week, in hanging metabolic cages for collection of 24-h urine samples. Urine was collected in dark, sterile containers for the measurement of [3H]TC content, as described above, by liquid scintillation counting.

Serum osteocalcin. Serum osteocalcin concentration was measured by radioimmunoassay before and after 9 wk with a goat anti-rat osteocalcin antibody (Biomedical Technologies, Stoughton, MA). For blood collection, rats were restrained using disposable rodent restrainers (DecapiCone, Braintree Scientific, Braintree, MA) and samples were collected from a lateral tail vein using a 0.5-mL tuberculin syringe (Monoject, Sherwood Medical, St. Louis, MO).

Dual energy x-ray absorptiometry (DXA). DXA of the whole body was performed before and after dietary intervention, using a Lunar DPX-L densitometer (Lunar, Madison, WI) and Small Animal Software (version 1.0) at a high resolution mode (slow speed for animals <0.5 kg). Before scanning, rats were anesthetized by intraperitoneal injection of a mixture of Ketaset (80 mg/kg) and Xylazine (12 mg/kg). The densitometer was calibrated daily.

Statistics. The effects of dietary calcium (low and high), energy (low and high) and age (younger and older) using all eight treatment combinations was assessed using a three-way analysis of variance (ANOVA) with repeated measures over time on measures of both body weight and urinary [3H]TC excretion. This was followed by a Scheffe’s post hoc comparisons test to determine statistical significance among the treatment groups (SuperANOVA, version 1.1, Abacus Concepts) (Zar 1984). For values measured only before and after dietary treatment (serum osteocalcin and bone mineral density), a three-way ANOVA was performed on the treatment combinations described above. An unpaired Student’s t test was used to assess the effect of the two age groups on [3H]TC incorporation into bones. Data are presented in text as mean values ± SD. Differences between means were considered significant at P < 0.05.

RESULTS

Tissue [3H] content. [3H]TC uptake was limited to skeletal tissue in both younger and older rats. Bones of older rats, however, incorporated less than half as much [3H] as bones of younger animals (44.3 ± 10.4 vs. 102.3 ± 12.0 kBq) (P < 0.01).

Body weight. During 9 wk of study, body weights of younger rats increased by 26.0 ± 7.6% and 23.0 ± 8.3% in CNTL and CR groups, respectively (P < 0.001, Table 2, Fig. 1). Younger energy-restricted rats (ER and CER), lost 9.0 ± 2.1% and 5.0 ± 1.8% of initial body weight, respectively (P < 0.001 compared to CNTL).

Among older rats, energy-adequate rats (CNTL and CR) increased body weight by 14.0 ± 2.6% and 11.0 ± 1.9%, respectively (P < 0.01), whereas energy-restricted rats (ER and CER) lost 21.0 ± 3.6% and 13.0 ± 1.9% of their initial body weight, respectively (P < 0.01).

Bone turnover. Dietary restriction of calcium (CR), energy (ER) or both (CER) elevated urinary [3H]TC excretion in both age groups to a similar degree above CNTL values (P < 0.01), followed by a return to CNTL levels after 6 wk (Fig.
TABLE 2

Initial and final body weight (BW) and bone mineral density (BMD) of rats fed control, calcium-restricted (CR), energy-restricted (ER) and calcium- and energy-restricted (CER) diets for 9 wk.1,2

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Control</th>
<th>CR</th>
<th>ER</th>
<th>CER</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Younger (3 mo)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial BW, g</td>
<td>269.3</td>
<td>270.1</td>
<td>268.4</td>
<td>268.2</td>
<td>21.4</td>
</tr>
<tr>
<td>Final BW, g</td>
<td>340.1a</td>
<td>332.2a</td>
<td>245.6b</td>
<td>256.5b</td>
<td>21.8</td>
</tr>
<tr>
<td>Initial BMD, g/cm²</td>
<td>0.271</td>
<td>0.273</td>
<td>0.270</td>
<td>0.268</td>
<td>0.003</td>
</tr>
<tr>
<td>Final BMD, g/cm²</td>
<td>0.293a</td>
<td>0.287b</td>
<td>0.294a</td>
<td>0.279b</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Older (10 mo)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial BW, g</td>
<td>325.2</td>
<td>324.7</td>
<td>322.4</td>
<td>324.2</td>
<td>15.8</td>
</tr>
<tr>
<td>Final BW, g</td>
<td>370.3a</td>
<td>360.3a</td>
<td>254.8b</td>
<td>283.6b</td>
<td>14.7</td>
</tr>
<tr>
<td>Initial BMD, g/cm²</td>
<td>0.299</td>
<td>0.303</td>
<td>0.301</td>
<td>0.301</td>
<td>0.002</td>
</tr>
<tr>
<td>Final BMD, g/cm²</td>
<td>0.309a</td>
<td>0.302b</td>
<td>0.300a</td>
<td>0.299b</td>
<td>0.002</td>
</tr>
</tbody>
</table>

1 Values are means for younger (n = 5–6) rats and older (n = 11–12) rats.
2 Within age groups, mean values within rows for each variable having different superscripts are significantly different (P < 0.05).

2). Combined restriction of calcium and energy had no additive effects on stimulation of urinary [3H]TC excretion. In younger rats, average urinary excretion of [3H]TC was 2.74 ± 0.29 kBq in CNTL and 3.09 ± 0.27, 2.98 ± 0.37 and 3.45 ± 0.24 kBq in CR, ER and CER, respectively. In older rats, average urinary excretion of [3H]TC was 2.86 ± 0.29 kBq in CNTL and 3.15 ± 0.318, 3.28 ± 0.22 and 3.62 ± 0.37 kBq in CR, ER and CER, respectively.

In both age groups, energy restriction (ER and CER), resulted in an elevated serum osteocalcin concentration, compared to CNTL (P < 0.05, Fig. 3). Calcium restriction alone (CR) had no effect on bone formation as measured by serum osteocalcin. Serum osteocalcin concentration increased over time in younger rats, approaching values observed in older rats (Fig. 3).

Bone mineral density. BMD, as assessed by DXA, increased 4–9% in all younger rats (P < 0.01, Table 2, Fig. 4). Dietary restriction of energy alone (ER) had no significant influence on final BMD compared with CNTL rats. Restriction of calcium (CR and CER), however, resulted in a significantly attenuated gain in BMD compared with CNTL (P < 0.01).

In older rats, BMD was increased 3.3 ± 2.6% in CNTL (P < 0.01), whereas no significant change in BMD occurred in any of the restricted groups. Consequently, final BMD in all diet-restricted groups was significantly lower (P < 0.05) than in CNTL (Fig. 4).

DISCUSSION

In this study, we demonstrated an elevated rate of bone resorption after dietary restriction of calcium or energy in both younger and older rats. Bone formation, however, was elevated only in energy-restricted groups. In both younger and older rats, final BMD was lower than control values after dietary restriction of calcium, whether alone or in combination with energy restriction. Older rats, however, also had a lower final BMD after dietary restriction of energy alone. These results suggest that although calcium or energy restriction increases bone turnover similarly in both younger and older rats, age-related differences exist for the influence of energy restriction on BMD.

The importance of calcium intake for the development and maintenance of peak bone density is well established. Inadequate calcium intake by both rats and humans, increases bone resorption (Egger et al. 1994, Shapses et al. 1995), decreases
BONE TURNOVER AND DENSITY IN FEMALE RATS

Body weight gain partially protects rats from developing osteopenia after ovariectomy (Roudebush et al. 1993), but such protection is limited to long bones, suggesting a weight-bearing effect of increased body weight (Wronska et al. 1987). In the present study, however, dietary restriction of calcium clearly altered the positive relationship between body weight and BMD. For example, older rats restricted in calcium only (CR) increased BW as did controls yet had a significantly lower final BMD. This suggests that calcium-deficient diets can abolish the positive relationship between body weight and bone mass at maturity. Although energy restriction and loss of body weight resulted in a lower final BMD compared with older controls, we only assessed BMD of the total body and therefore cannot draw any conclusions regarding site-specific BMD. It has been suggested, however, that calcium or energy restriction may result in a redistribution of calcium stores from one skeletal site to another (Garcia-Moreno et al. 1995).

Complete food deprivation and semistarvation in rats reduces osteoblastic activity (Wright and McMillan 1994); bone mass (Matkovic et al. 1990, Persson et al. 1993) and increases the risk of osteoporosis (Heaney 1996).

The skeletal effect of reduced energy intake is less clear, particularly in rapidly growing younger animals. There is some data in aged rats, however, to suggest that despite adequate calcium intake, energy restriction leads to a loss of bone mineral content (Lee et al. 1986). The present study is the first examination of the interaction between calcium and energy restriction that also examines age-related effects. The ages examined in the present study, 3 and 10 mo, were chosen to avoid the large amount of bone modeling and skeletal growth characteristic of very young rats (<2 mo old) (Kalu 1991) and to ensure the achievement of peak bone mass in older rats, which occurs at ~10 mo in female rats (Kimmel 1994). When calcium intake was maintained, we found no effect of energy restriction on BMD in younger rats. Our data in older rats, however, support the hypothesis that dietary energy restriction, with or without adequate calcium intake, decreases total body BMD compared with a calcium- and energy-adequate control diet.

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Complete food deprivation and semistarvation in rats reduces osteoblastic activity (Wright and McMillan 1994); bone mass (Matkovic et al. 1990, Persson et al. 1993) and increases the risk of osteoporosis (Heaney 1996).

FIGURE 2 Stimulation of urinary tritiated tetracycline ([3H]TC) excretion in younger (A) and older (B) rats by dietary restriction of calcium (CR), energy (ER) or calcium and energy (CER). Each line represents 5–6 or 10–12 rats per diet group for the younger and older groups, respectively. Data are reported as mean percent of control values (pooled SEM = 8.7% for younger rats and 6.2% for older rats). Urinary [3H]TC excretion for CR, ER and CER was elevated above control values at wk 2–6 (P < 0.05) in both age groups.

FIGURE 3 Serum osteocalcin concentrations of younger (A) and older (B) rats before and after 9 wk of dietary restriction of calcium (CR), energy (ER) or both calcium and energy (CER). Each bar represents the mean ± SEM of 5–6 or 10–12 rats per diet group for the younger and older groups, respectively. Bars labeled with different letters are significantly different at P < 0.05.
formation (Ndiaye et al. 1992) and overall bone turnover and bone mass (Shires et al. 1980) and attenuates the normal age-associated increase in serum calcitonin (Kalu et al. 1983). Additionally, Muhlbauer and Fleisch (1995) have demonstrated that the increase in bone resorption after feeding can be blunted by meal portioning. Although results from these studies provide support that total food intake is an important regulator of bone turnover and bone mass, the designs did not specifically control calcium intake during energy restriction or address whether the effect on bone differs with age.

In a study of reduced energy intake by rats in which dietary calcium was controlled, a reduction in bone mass occurred whether such deficits were continuous or cyclic in nature (Lee et al. 1993), suggesting that either chronic or transient periods of energy restriction are detrimental to skeletal health. Although Ndiaye et al. (1995) found that 60% energy restriction reduced serum osteocalcin concentration, no change was noted after 40% restriction. In the present study, however, we found an elevated serum osteocalcin concentration after 40% energy restriction. The apparent discrepancy in serum osteocalcin results between the present study and those of Ndiaye et al. (1995) may be explained by differences in study design. We examined both 3- and 10-mo-old rats during a 9-wk period, while Ndiaye studied weanling rats for 4 wk. From these results, it appears that 40% energy restriction is not severe enough to significantly reduce serum osteocalcin concentration in rats at any stage of development (weanling, younger or older).

A limitation of the present study is that we did not obtain serial measurements of serum osteocalcin to identify both the acute and chronic adaptation to dietary restriction. Because we observed an initial increase in bone resorption during weeks 2–6 followed by a return to control levels thereafter, our finding of increased serum osteocalcin at wk 9 could be viewed as a "delay" between resorption and formation phases. These data would support those of Sims et al. (1996), who demonstrated a delay in bone formation (serum osteocalcin) after ovarectomy-induced bone resorption (acid phosphatase activity) in 6-mo-old rats. A future study obtaining serial measures of both bone resorption and formation could help elucidate the relationship between dietary intake and bone turnover.

In summary, these data indicate that in both younger and older female rats, dietary restriction of calcium or energy results in an elevated rate of bone turnover compared with a calcium- and energy-adequate control diet. In both younger and older rats, reduced BMD was observed after dietary calcium restriction. Additionally, energy restriction in older rats also resulted in a blunted increase in BMD, suggesting an adverse effect not only of low calcium but also of low energy diets on skeletal health in mature animals. This work raises concerns that dietary energy restriction may have unfavorable effects on bone metabolism in humans. Further studies are needed to address the mechanisms by which energy restriction increases bone turnover and why the response of bone to dietary restriction differs with age.

**LITERATURE CITED**


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**FIGURE 4** Percent change in bone mineral density of younger (A) and older (B) rats before and after 9 wk of dietary restriction of calcium (CR), energy (ER) or both calcium and energy (CER). Each bar represents the mean percent change ± SEM from initial to final BMD of 5–6 or 10–12 rats per diet group for the younger and older groups, respectively. Bars labeled with different letters are significantly different at P < 0.05.


