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Moderate Weight Gain Does Not Influence Bone Metabolism in Skeletally Mature Female Rats

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Abstract

Bone mass is correlated with body weight during growth. However, it is unclear how bone mass is influenced by weight gain following skeletal maturity. The purpose of this study was to determine the effects of weight maintenance and two rates of weight gain on bone metabolism using skeletally mature female rats. Eight-month-old female rats were fed one of 3 diets for 13 weeks: Lieber DeCarli liquid diet *ad libitum* (control diet), the same diet with caloric restriction to maintain initial body weight (calorie-restricted diet), and the same diet fed *ad lib* with the exception that appetite was enhanced (calorie-increased diet) by replacing a small quantity of maltose-dextran isocalorically with ethanol (0.5% caloric intake). Compared to baseline, rats fed the calorie-restricted, control, and calorie-increased diets changed in weight by $-1 \pm 2\%$ (mean $\pm$ SE), $10 \pm 3\%$, and $21 \pm 2\%$, respectively. Weight gain was associated with a significant increase in serum leptin, a putative regulator of bone formation. In contrast, significant differences in tibial bone mineral content and density were not detected among treatments groups following dietary intervention or between treatment groups and the baseline group. Similarly, indices of cancellous bone architecture (area, trabecular number, thickness, and separation) and bone turnover (mineralizing perimeter, mineral apposition rate, and bone formation rate) did not differ among groups following dietary intervention. Our findings suggest that neither weight gain nor increased serum leptin levels, over the range evaluated, influence bone metabolism in skeletally mature female rats.

Keywords

Bone turnover; caloric restriction; osteoporosis; obesity; leptin

Introduction

The peak prevalence of obesity in adults is reached between 50 and 60 years of age [1,2]. Excessive weight gain in this population is associated with a variety of chronic diseases and increased mortality. Obese individuals are at increased risk for heart disease, chronic obstructive pulmonary disease, type 2 diabetes, osteoarthritis, and certain cancers [3–5].

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Not all health effects correlated with excessive weight gain are detrimental. Obesity in humans is often associated with increased bone mass and reduced risk for osteoporosis [6,7]. Bone mass is also correlated with body weight in growing mice fed a normal diet [8]. The mechanisms for the positive relationship between body weight and bone mass are under investigation. Increased skeletal loading may be a contributing factor but obese individuals often have increased bone mass at non-weight bearing as well as weight bearing sites [9]. Also, *ob/ob* mice, in spite of being morbidly obese due to inability to produce the adipokine leptin, have smaller bones with locally increased trabecular density than their lean wild type counterparts [10–13]. Thus, the specific contributions of body weight, energy intake compared to expenditure, and circulating levels of adipokines on bone balance remain unclear.

Female rats increase in body weight following skeletal maturity, providing an opportunity to investigate the effects of weight gain on the non-growing skeleton [14,15]. In rats, longitudinal growth ceases as a result of formation of bone bridges that penetrate and cross the growth plate. This occurs by 8 months of age in tibia of female Sprague Dawley rats [14]. As in adult humans, radial bone growth in rats continues at a very slow rate following cessation of longitudinal bone growth [14,15]. Also, in rats, as in humans, cancellous bone remodeling continues throughout adult life [16].

The goal of the present study was to determine whether increases in weight have an effect on bone mass, architecture, and turnover in skeletally mature 8-month-old female rats. This goal was accomplished by comparing the skeletal effects of 13 weeks of treatment using 3 dietary interventions: rats were caloric restricted to prevent further weight gain, fed the same diet *ad lib* to monitor normal weight gain, or fed the same diet slightly modified by isocaloric replacement of a small amount (0.5 % caloric intake) of maltose/dextran with alcohol to stimulate appetite and increase food consumption. Additionally, we evaluated accompanying changes in serum leptin levels as a potential mechanism for the anticipated effects of weight gain on bone metabolism.

Methods

Animal experiment

Eight-month-old, virgin, female Sprague-Dawley rats weighing 279 ± 3 g (mean $\pm$ SE) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). The rats were housed individually in a temperature- and humidity-controlled animal facility on a 12-h light/dark cycle. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

During the first week of the study, all animals were acclimated to a modified Lieber-DeCarli liquid diet (BioServe, Frenchtown, NJ), the use of which facilitated administration of alcohol and measurement of food consumption. The diet contained 1.3 g/liter calcium and 1.7 g/liter phosphorous. Protein, fat, and carbohydrates contributed 18, 35, and 47% of caloric intake, respectively.

Following acclimation, the rats were randomized by weight into four groups ($n = 10$ /group); 1) a group sacrificed at treatment initiation (baseline), 2) a group fed the diet *ad lib* (control), 3) a group in which the amount of diet was restricted to prevent weight gain (calorie-restricted), and 4) a group fed the diet *ad lib* in which ethanol contributed 0.5% of caloric intake to induce increased food consumption (calorie-increased) [17]. In the calorie-increased group, alcohol isocalorically replaced maltose/dextran. The volume of food offered to the rats in the calorie-restricted group was adjusted daily to minimize changes in

body weight. The rats consumed their respective diets for 13 weeks. All but baseline control rats were injected (20 mg/kg body weight) with the fluorochromes calcein (Sigma, St. Louis, MO) and demeclocycline (Sigma) 9 and 2 days before sacrifice, respectively, to label mineralizing bone.

For tissue collection, the rats were anesthetized using ketamine and xylazine, weighed, and decapitated. Trunk blood was collected for measurement of serum leptin and tibiae were harvested and fixed in 70% ethanol for bone dual energy absorptiometry (DXA) and histomorphometry.

Serum Leptin

Serum leptin was measured as a biochemical marker of fat mass by immunoassay as recommended by the manufacturer (R&D Systems, Minneapolis, MN).

DXA

Whole tibiae with attached fibulae were scanned *ex vivo* using DXA (PIXIMUS, Lunar Corporation, Madison, WI) to determine bone mineral content (BMC, mg), apparent tissue area of the bone and marrow, and bone mineral density (BMD, mg/cm²).

Histomorphometry

For histomorphometric evaluation of cancellous bone, proximal tibiae were dehydrated in a graded series of ethanol and xylene, and embedded undecalcified in modified methyl methacrylate. Longitudinal sections (5 µm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to slides. One section/animal was used for determining bone area and fluorochrome-based measurements of bone formation.

Histomorphometric data were collected at 10x within the proximal tibial metaphysis using the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA) as described [18]. The region of interest (2.9 mm²) included cancellous bone only. Static cancellous bone measurements included: 1) cancellous bone area/tissue area (area of total tissue evaluated that is occupied by cancellous bone, %), 2) trabecular thickness (µm), 3) trabecular number (1/mm), and 4) trabecular separation (µm). Fluorochrome-based indices of bone formation included: 1) mineralizing perimeter/bone perimeter (percentage of cancellous bone perimeter covered with double fluorochrome label, %), 2) mineral apposition rate (distance between two fluorochrome labels that comprise a double label divided by the number of days between label administration, µm/day), and bone formation rate (calculated using a perimeter referent (µm²/µm/day) as well as a bone area referent (%/year) as described [19]. All bone histomorphometric data are reported using standard nomenclature [20].

Statistics

Differences among treatment groups were determined using one-way ANOVA (SPSS 17.0, SPSS Inc., Chicago, IL). When significant treatment effects were detected, a Bonferroni post-hoc test was used to evaluate pair-wise differences. If ANOVA assumptions of homogeneity of variance were not met, a Kruskal-Wallis test followed by a Tamhane post-hoc test was applied. A paired t-test was used to compare baseline and terminal body weight within a treatment group. Differences were considered significant at $P < 0.05$. All data are reported as mean $\pm$ SE.

Results

The effect of dietary interventions on body weight as a function of time is shown in Figure 1A. Caloric restriction was effective in preventing body weight gain. Compared to initial

values, rats in the calorie-restricted group did not change significantly in body weight ($-1 \pm 2\%$) during the 13 week duration of study. In contrast, rats in the control group increased in weight by $10 \pm 3\%$ ($P < 0.006$) and those in the calorie-increased group increased in weight by $21 \pm 2\%$ ($P < 0.001$) compared to initial values. At the end of the dietary intervention, the rats in the calorie-increased group were 15% ($P < 0.001$) heavier than rats fed the control diet.

Food intake as a function of time for the 3 treatment groups is shown in Figure 1B. The quantity of food required to maintain stable body weight in the calorie-restricted group was $19.8 \pm 1.5\%$ ($P < 0.001$) less than that consumed by the control group. Rats fed a diet containing a small amount of alcohol (calorie-increased) consumed $20.4 \pm 1.8\%$ ($P < 0.001$) more diet than rats in the control group. The total volume of diet consumed during the 13 week intervention by the calorie-restricted, control, and calorie-increased groups was $4,927 \pm 89$, $5,903 \pm 105$, and $7,109 \pm 106$ ml, respectively.

The effect of dietary intervention on serum leptin levels is shown in Figure 2. Compared to baseline, leptin levels were not significantly changed in the calorie-restricted group, and significantly higher in the control diet and calorie-increased diet groups by $183 \pm 46\%$ ($P < 0.028$) and $607 \pm 77\%$ ($P < 0.002$), respectively. At the end of the dietary intervention, the rats in the calorie-increased diet group had serum leptin levels that were $150 \pm 27\%$ ($P < 0.008$) greater than the rats fed the control diet.

The effect of intervention on total tibia BMC and BMD is shown in Table 1. Tibial BMC and BMD in control rats sacrificed at baseline did not differ from the treatment groups sacrificed following the 13 weeks of dietary intervention. Furthermore, differences in BMC or BMD among treatment groups were not detected following dietary intervention.

The effects of treatments on static and dynamic bone histomorphometry of the proximal tibial metaphysis are shown in Table 2. Significant differences among treatment groups were not detected for cancellous bone area/tissue area, trabecular number, trabecular thickness, trabecular separation, mineralizing perimeter/bone perimeter, mineral apposition rate, or bone formation rates expressed relative to either bone perimeter or bone area referents.

Discussion

Compared to *ad lib* feeding the standard diet, weight gain was prevented by caloric restriction and enhanced by increasing food intake through the addition of a small amount of alcohol in the diet. Body weight of the rats in the calorie-restricted, control, and calorie-increased groups changed compared to initial values by $-1 \pm 2\%$, $10 \pm 3\%$ and $21 \pm 2\%$, respectively. A similar pattern among groups was observed for energy intake and serum leptin levels. In spite of the large differences in energy consumption and weight gain among treatment groups, significant intergroup differences were not detected in indices of bone mass, architecture, or dynamic histomorphometry.

The present studies evaluated BMC and BMD of whole tibia and cancellous bone mass, architecture and turnover in proximal tibial metaphysis of skeletally mature rats. The tibia is a weight bearing bone and can adapt to changes in mechanical loading. However, the effects of loading on bone are non-linear. Consistent with the mechanostat theory, bone responds to changes in muscle forces once a threshold level of loading is crossed [21,22]. Large reductions in skeletal loading during disuse (e.g., spaceflight or immobilization) decrease bone formation and increase bone resorption [23,24]. This uncoupling between formation and resorption results in rapid bone loss [25,26]. However, increasing loads on the skeleton by up to 65% using weighted backpacks had no effect on cortical or cancellous bone histomorphometry or bone biomechanical properties in 12-month-old female rats [27]. This

lack of an effect in normal rats was likely due to the failure to increase loads sufficient to induce an adaptive response. In comparison, simple jumps in humans generate ground reaction forces 3–5 times resting body weight, and 45 minutes of such activity/day results in rates of force that are 500 times greater than body weight [28]. Thus, based on the magnitude of the changes in body weight observed in the present study, it is not entirely surprising that bone mass did not differ among treatment groups.

There is discussion in the literature as to whether bone mass should be adjusted to body weight and if so whether it should be adjusted to total or lean mass [29–31]. In the present study, the amount of bone mass per unit body weight decreased in the two *ad lib* fed groups compared to the calorie-restricted group by an amount proportional to the increase in body weight. Adjustment of bone mass to body weight implies that there is a consistent causal relationship between the two endpoints. However, the weight of evidence does not support a tight relationship. BMC and BMD typically increase with bodyweight up to skeletal maturity, but the present study suggests that the relationship is much less pronounced in adults. Even in growing animals, the relationship between bone mass and body weight is neither linear nor constant among skeletal sites. In inbred mice fed a normal diet, differences in body weight explain 35–75% of the variation in bone mass. Furthermore, the precise relationship between body weight and bone mass depends upon the individual bone (e.g., femur versus 3rd lumbar vertebra) and region (diaphysis versus metaphysis) measured [8]. These observations argue strongly against indiscriminant normalization of bone mass to body weight as an index of bone health.

Increased weight gain in the present study was associated with increased serum leptin levels. The adipokine leptin is produced in proportion to fat mass and serum levels of the hormone reflect energy stored in fat [32]. Leptin plays a key role in energy homeostasis by influencing food consumption and non-shivering thermogenesis [33,34]. However, the rise in leptin levels observed in the present studies had no clear cut effect on food consumption or weight gain.

There is evidence that bone metabolism is coupled to energy homeostasis through leptin signaling [35,36]. In support of a critical role for leptin signaling, leptin-deficient *ob/ob* mice have multiple skeletal abnormalities which are corrected by systemic or central leptin administration or by hypothalamic leptin gene therapy [12]. *ob/ob* mice and leptin receptor-deficient mice (*db/db*) and rats (Zucker *fa/fa*) are morbidly obese but, even without adjusting for the increase in body weight, have low bone mass compared to lean littermates [8,36,37]. These findings in leptin signaling-deficient rodents suggest that leptin may play a role in mediating the increase in bone mass associated with excessive weight gain. However, studies performed in lean wild type and *ob/ob* mice fed normal and high fat diets illustrate that body weight gain has both independent, as well as leptin-dependent, effects on bone mass and architecture [8].

Although leptin is essential for normal bone growth and turnover, similar to its action on reproduction [38], the hormone may act on the skeleton as a permissive off/on factor. Whereas administration of leptin to leptin-deficient *ob/ob* mice has a profound effect on bone metabolism, there is a notable lack of an effect in leptin-replete animals [39,40]. The results of the present study further support the conclusion that the skeletal actions of leptin are largely permissive because substantial increases in body weight with correspondingly large increases in serum leptin levels within the physiological range had no effect on bone mass, architecture, or turnover in skeletally mature female rats.

Increases in body weight can be induced in rodents by feeding them energy dense high fat diets. Bone compartment-specific increases and decreases in bone mass have been reported

in growing mice fed high fat diets [8,41]. A limitation of comparing high and normal fat diets is that it is difficult to distinguish between the effects of weight gain and those caused by differences in composition of the diets. In the present study, the major cause for the differences in weight gain among treatment groups was the volume of food consumed. The diet of the calorie-increased group contained ethanol, which in total contributed only 0.5% of caloric intake. Alcohol stimulates appetite in humans [42,43]. Whereas high concentrations of alcohol suppress appetite in rats [19], the small amount used in the present study significantly increased the volume of food consumed and facilitated body weight gain. This finding was anticipated because we had noted increased food consumption with low levels of alcohol consumption in dose-response studies in rats [17].

Alcohol itself has potent effects on bone metabolism. In adult female rats, dose-response studies revealed that levels of alcohol consumed by moderate drinkers (3 and 6% caloric intake) reduced bone turnover without impacting bone mass [17]. However, we found that alcohol consumption of 1 or 2% caloric intake had no significant effect on bone histomorphometry (unpublished results). Thus, it is unlikely that the even lower level of alcohol used to stimulate appetite in the current study had independent effects on bone metabolism.

Dietary restriction, resulting in reduced weight gain during growth [44], or a reduction in body weight in adults loss [45–49], is accompanied by decreased bone formation and/or increased bone resorption. Since bone formation requires energy, it is not surprising that reduced bone formation occurs when energy is severely restricted. Although there were marked differences in food intake, serum leptin levels, and body weight among treatment groups in the current study, even the calorie-restricted group was fed sufficient energy to maintain body weight. Thus, the present study demonstrates that weight gain, over the range studied, in skeletally mature female rats had neither beneficial nor detrimental effects on bone mass, architecture, or turnover. Furthermore, the results do not support a role for serum leptin, over the range evaluated, in regulating bone metabolism in skeletally mature female rats.

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Figure 1.

Effect of diet on body weight (A) and food consumption (B) as a function of time in 8-month-old female rats. The inset in Figure 1B represents total food consumed over the 13-week duration of study. Groups with different letters are different ($P < 0.05$) from one another. *Different from baseline, $P < 0.05$. Values are mean $\pm$ SE.

Figure 2. Effect of diet on serum leptin levels at baseline and following 13 weeks of dietary intervention. Groups with same letter are not different ($P > 0.05$) from one another. Values are mean $\pm$ SE.

Table 1

Effect of body weight on tibial BMC and BMD.

Endpoint	Diet				ANOVA (P<)
	Baseline	Control	Calorie-restricted	Calorie-increased	
Bone mineral content (mg)	0.34 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	NS
Bone mineral density (mg/cm ²)	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	NS

Data are mean ± SE

Table 2

Effect of body weight on bone histomorphometry in the proximal tibial metaphysis.

	Diet			ANOVA (P<)
	Control	Calorie-restricted	Calorie-increased	
Static Endpoints				
Bone area/Tissue area (%)	16.3 ± 2.5	17.1 ± 1.6	14.6 ± 2.5	NS
Trabecular number (1/mm)	2.7 ± 0.2	3.0 ± 0.1	2.6 ± 0.2	NS
Trabecular thickness (μm)	57 ± 6	55 ± 3	55 ± 4	NS
Trabecular separation (μm)	343 ± 58	282 ± 20	341 ± 34	NS
Dynamic Endpoints				
Mineralizing perimeter/Bone perimeter (%)	8.2 ± 1.3	6.7 ±0.7	6.4 ± 1.2	NS
Mineral apposition rate (μm/day)	0.76 ± 0.06	0.75 ± 0.03	0.72 ±0.04	NS
Bone formation rate/Bone perimeter (μm ² /μm/day)	0.062 ± 0.010	0.052 ± 0.006	0.046 ± 0.009	NS
Bone formation rate/Bone area (%/day)	0.22 ± 0.03	0.19 ± 0.02	0.19 ± 0.05	NS

Data are mean ± SE

NS, Not significant