Blocking β-adrenergic signaling attenuates reductions in circulating leptin, cancellous bone mass, and marrow adiposity seen with dietary energy restriction

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We previously demonstrated in a side-by-side comparison with equivalent calcium and protein restriction that reduced energy intake is the major contributor to reductions in bone mineral density and serum estrogen levels seen with restriction of all nutrients (global food restriction) (2). In addition, negative energy balance in humans and animals is associated with higher bone marrow adipogenesis, which often appears coincident with lower bone mass (10, 17).

A fascinating line of research has emerged over the last decade detailing the role the sympathetic nervous system (SNS) plays in the regulation of bone mass. SNS activation has been shown to reduce osteoblast proliferation (43) and to stimulate bone resorption by increasing RANKL expression (19); over time, this can produce a loss of cancellous bone mass. Increased SNS activation may also play a role in ER-induced bone loss. Global food restriction can also increase norepinephrine release (47), which is associated with increased bone resorption and decreased bone formation. Pharmacological β-blockade mitigates loss of cancellous bone in the context of stressful conditions such as disuse via hindlimb unloading in mature rats (4), ER in adult mice (45), and ovariectomy in exercising adult rats (7). Several lines of evidence support SNS regulation of osteoblast function. Takeda et al. confirmed that osteoblasts express β-adrenergic receptors (43). Wild-type mice given a β-adrenergic antagonist experience an increase in bone mass. Leptin-deficient mice (ob/ob) have low sympathetic activity but high vertebral bone mass; if a β-adrenergic agonist (isoproterenol) is administered, bone mass declines by 45% without affecting appetite (43). In addition, β2-adrenergic receptor knockout mice have a high bone mass phenotype but no other endocrine abnormalities that affect bone metabolism (19).

Leptin, a cytokine-like hormone principally produced by white adipocytes, is one of the important endocrine factors altered by ER. Although its principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic nuclei, leptin also has multiple effects on bone cell differentiation and function. The nature of leptin’s effects on bone is somewhat controversial. Leptin that binds to its hypothalamic receptors demonstrates anti-osteogenic effects via the SNS signaling (18, 43), but when physiological doses of leptin are administered peripherally, leptin exerts a bone protective effect by mitigating bone loss due to mechanical unloading, ovariectomy, or ER (4, 11, 21). Expression of leptin receptors on the cells of osteoblastic lineage has been demonstrated, confirming that osteoblasts are targets for leptin action (16, 40, 46). In addition to its positive effect on osteoblast differentiation (16), leptin inhibits the expression of RANKL, synthesized by osteoblasts and an important stimulator of bone resorption, as well as enhances the expression of OPG, the decoy receptor for RANKL (27).

AT ANY GIVEN TIME, >50% of U.S. women are restricting dietary calories in attempts to lose weight (52). Chronic dietary restriction of energy intake can lead to significant clinical concerns such as impaired hypothalamic-pituitary-ovarian signaling and decreased bone mass (29). There is a clear correlation between a history of weight loss in premenopausal years and increased hip fracture risk later in life (35). On average, each 10% decrement in body weight in humans is associated with a 1% decrease in bone mass (41, 42). Consistent bone loss associated with energy restriction (ER) has been observed in animal models (14, 44) as well as in humans (29, 31, 41). There is a rich literature reporting the positive effects of controlled ER (with other nutrients supplemented) with regard to extending the lifespan (51) but fewer published studies describing the deleterious impact of ER on bone mass.

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We previously demonstrated that hindlimb unloading and global food restriction each independently produce a decrease in serum leptin, with the combined treatment resulting in undetectable serum leptin levels (3). When propranolol, a β-adrenergic receptor antagonist, is administered to hindlimb-unloaded rats, this blockade of β-adrenergic signaling attenuates the unloading-induced decline in serum leptin and reductions in cancellous bone mass and bone formation rate (3). However, it remains unproven whether blocking β-adrenergic signaling attenuates ER-induced bone loss and increases in marrow adiposity and whether these effects are associated with concomitant mitigated reductions in circulating leptin. In the present study, we tested 1) whether β-adrenergic blockade attenuates decreases in serum leptin and bone mass and increases in marrow adiposity during dietary ER and 2) whether rescued circulating leptin is associated with this bone-loss attenuation.

MATERIALS AND METHODS

Animals and experimental design. Forty Sprague-Dawley virgin female rats, aged 4 mo at purchase (Harlan, Indianapolis, IN), were singly housed on a 12-h light-dark cycle and allowed to eat AIN93-M purified diet (Research Diets, New Brunswick, NJ) for 8 wk before the start of the experiment. The AIN93-M diet most closely matches National Research Council (NRC) recommendations for mature rat dietary requirements (38, 39). Because we had previously observed a significant loss in volumetric bone mineral density (vBMD) in metaphyseal bone in adult rats switched from the chow fed by the vendor (Harlan Teklad 2018) to this AIN93-M diet, we tested an extended period of acclimation to this diet with weekly computed tomography scans. A stable proximal tibia vBMD (defined by two consecutive average vBMD values that change by <10 mg/cm²) was observed by 6 wk; we chose to extend this dietary acclimation period to 8 wk.

Experimental design. Rats were block assigned first by cancellous vBMD at the end of acclimation and then by body weight into four groups of 10 animals each: ad libitum-fed controls (vBMD at the end of acclimation and then by body weight into four attenuations. Rescued circulating leptin is associated with this bone-loss attenuation.

### Table 1. AIN-93M rodent diet and modifications for 40% energy restriction diet

<table>
<thead>
<tr>
<th>Macronutrient, %kcal</th>
<th>AIN-93M</th>
<th>AIN-93M-E, 40% Energy Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>76</td>
<td>60</td>
</tr>
<tr>
<td>Fat</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>kcal/g</td>
<td>3.85</td>
<td>3.76</td>
</tr>
<tr>
<td>Mineral and vitamin, g/kg diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>3.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Calcium</td>
<td>5.00</td>
<td>8.10</td>
</tr>
<tr>
<td>Potassium</td>
<td>3.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>0.00075</td>
<td>0.00122</td>
</tr>
<tr>
<td>Vitamin D, IU/kg diet</td>
<td>1000</td>
<td>1621.3</td>
</tr>
</tbody>
</table>

(maximum volume 1.5 ml) while the rat was anesthetized for pQCT scans, and serum was saved at −80°C. On days 9 and 2 before death, animals were given subcutaneous injections of calcine (25 mg/kg) to label mineralizing bone for histomorphometric analyses. Left femora were fixed in 4% paraformaldehyde for immunohistochemistry (IHC) staining. Left tibiae were stored in 70% ethanol at 4°C for histology. All procedures in this study were approved by Texas A&M Institutional Animal Care and Use Committee.

Peripheral computed tomography and DEXA. The XCT Research M (Stratec, Norland, Fort Atkinson, WI) pQCT scanner has a scanning beam thickness of 500 μm and was calibrated daily using a standard hydroxyapatite phantom. In vivo scans using a voxel size of 100 μm were taken at the proximal tibia metaphysis with three transverse images at 5.0, 5.5, and 6.0 mm from the tibia plateau and from mid-shaft tibia at 50% total tibial length. A standardized analysis for either metaphyseal bone (contour mode 3, peel mode 2, outer threshold of 0.169 g/ml, inner threshold of 0.650 g/ml) or diaphyseal bone (separation 1, threshold of 0.650 g/ml) was applied to each slice. Values of total, cortical shell, and cancellous volumetric bone mineral density (vBMD) and cross-sectional total, cortical, and marrow areas were averaged across all slices to yield a mean value for each site. Machine precision (based on manufacturer’s data) is ±3 mg/cm² for cancellous vBMD and ±9 mg/cm² for cortical BMD. Reproducibility in our laboratory for in vivo measures was determined from five repeat scans with repositioning of the animal between scans. Coefficients of variation for these measurements were ±1.24, 2.13, and 1.95% for in vivo proximal tibia total BMD, cancellous BMD, and total area, respectively.

Total BMD, bone mineral content (BMC), fat mass, and lean mass were measured by DEXA with a Lunar DPX-MD + bone densitometer (GE Lunar, GE Medical Systems, Milwaukee, WI), with software standardized for small animals. Anesthetized rats were scanned while positioned prone on the platform; the tail was not included in total body scan. Daily densitometer standardization with a phantom spine varied by <0.1%.

Bone histomorphometry. Undemineralized distal left femora were subjected to serial dehydration and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were cut 8 μm thick and left unstained for fluorochrome label measurements; 4-μm sections were treated with Von Kossa stain for measurement of cancellous bone volume and quantification of osteoblast, osteoid, and osteoclast surfaces. Histomorphometric analyses were performed with Os teoMeasure system (OsteoMetrics, Atlanta, GA) interfaced with a color video camera (DXC-390P, Sony) and an Olympus BX60 Microscope with epifluorescent light (Leeds Instruments, Irving, TX). At ×20, a defined region of interest was established ~1.0 mm from the growth plate and within the endocortical edges encompassing 6–7 mm². Total bone surface, single-labeled surface, and double-labeled surface were measured at ×100; interlabel distances, bone volume,
and osteoid/osteoclast surface were measured at ×200. Mineral ap-
position rate (MAR; μm/day) was calculated by dividing the average 
time by the time between labels (7 days), and mineralizing 
surface (MS/BS) for cancellous bone surfaces was calculated by using 
the formula MS/BS = [(single labeled surface/2) + double label 
surface/surface perimeter] × 100. Bone formation rate was cal-
culated as MAR × MS/BS. Total bone, osteoid, and osteoclast sur-
faces were obtained by manual tracing. Derived indexes of bone 
volume/tissue volume referent (BV/TV), osteoid surface/bone surface 
(Os/BS), osteoclast surface/bone surface (Oc/BS), and osteoid thick-
ness (Os.Th) were calculated using previously described formulae (3). 
Nomenclature for cancellous histomorphometry follows standard us-
age (36).

The number of marrow adipocytes was assessed, referring to 
standard bone marrow adiposity quantification protocol (17, 22). 
Briefly, the number of adipocytes was manually counted at ×20 in a 
region of interest within the proximal femur beginning 1 mm distal 
the growth plate and within the endocortical edges, encompassing a 
total of 6–7 mm². The number of adipocytes and osteoblastic cells 
positively stained for leptin were manually counted at ×1100. 
Assays were run on duplicate samples. Precision coefficient of vari-

was used to measure the concentration of leptin in serum samples. 
antibody [Ob (A20): sc-842, Santa Cruz Biotech, Santa Cruz, CA], 
incubated overnight at 4°C with primary rabbit polyclonal anti-leptin 
solution and then biotin solution for 15 min each, then with normal 
being washed in PBS, the sections were pre-incubated with avidin D 
room temperature to block endogenous peroxidase activity. After 
incubated in 0.3% H2O2 in distilled de-ionized water for 30 min at 
(28); only the highest quality sections could be used for these 
ancies, reducing the number of samples analyzed to four or 
five per group. Osteoblastic cells were defined as mononuclear cells 
ading to a trabecular bone surface (including osteoid); most ap-
ppeared to be either active osteoblasts or flatter bone lining cells (which 
are often described as quiescent osteoblasts). This excludes oste-
oclasts or other large marrow cells.

**Immunohistochemistry and serum leptin analyses.** Femur spec-
imens from rats were fixed by immersion in 4% paraformaldehyde in 
0.1 M PBS, pH 7.4. After 20–24 h of fixation, samples were 
decalcedified with 50% formic acid (by mixture with 20% sodium 
citrate, 1:1 ratio), then dehydrated in a graded series of ethanol and 
embedded in paraffin (Paraplast). Sections of the proximal femur (5 
μm thick) were immunostained for leptin by avidin-biotin technique 
(28); only the highest quality sections could be used for these 
ancies, reducing the number of samples analyzed to four or five per 
group. Tissue sections were deparaffinized, hydrated in PBS, and 
incubated in 0.3% H2O2 in distilled de-ionized water for 30 min at 
the same size region of interest within the proximal femur; only the 
highest quality sections (with no tearing or folding) could be used for 
theses analyses, reducing the number of samples analyzed to four or 
five per group. Tissue sections were deparaffinized, hydrated in PBS, and 
incubated in 0.3% H2O2 in distilled de-ionized water for 30 min at 
room temperature to block endogenous peroxidase activity. After 
being washed in PBS, the sections were pre-incubated with avidin D 
solution and then biotin solution for 15 min each, then with normal 
serum from the same species as the secondary antibody for 20 min at 
room temperature (to minimize background staining). Then they were 
incubated overnight at 4°C with primary rabbit polyclonal anti-leptin 
[Ob (A20): sc-842, Santa Cruz Biotech, Santa Cruz, CA], 
diluted 1:500 in PBS/2% BSA, then with the corresponding biotinyl-
ated anti-rabbit IgG secondary antibody, made in goat, diluted by 
manufacturer’s protocol in PBS/2% BSA/5% normal goat serum 
(NGS), and finally with ABC complex (avidin-biotin peroxidase 
complex) (Vectastain elite ABC reagent, VECTOR). Peroxidase ac-
activity was revealed by developing sections with enzyme substrate 
(NovaRED substrate kit, VECTOR). Sections were then counter-
stained with hematoxylin and examined for cells positively expressing 
leptin. Method specificity was tested by omitting the primary antibody 
in the immunostaining procedures as a negative control.

A rat leptin ELISA immunoassay kit (Crystal Chem, Chicago, IL) 
was used to measure the concentration of leptin in serum samples. 
Assays were run on duplicate samples. Precision coefficient of vari-
ation within run was ±3.2%, and coefficient of variation between run 
was ±6.4%.

**Statistical analysis.** To analyze pre- and posttreatment values of 
pQCT variables and serum leptin, a three-way ANOVA with repeated 
measures on time was used. In addition, a simple main-effects anal-
ysis within treatment group was performed when three-way interac-
tions were significant and, when appropriate, Duncan post hoc or least 
significant difference tests were used within the simple main-effects 
alyses. To analyze the delta values (pre-post) of tibia pQCT vari-
ables and serum leptin and variables measured only at the end of the 
experiment (e.g., histomorphometric parameters), two-way ANOVA 
was used with post hoc testing as appropriate. Linear associations 
between change in serum leptin to the bone formation rate measured 
at the end of the experimental period were tested with Pearson’s 
correlation coefficients. All values reported are means ± SE.

**RESULTS**

Over the course of the 12-wk study, CON rats (ad libitum-
fed VEH and BB) consumed 15.3 ± 2.7 g/day of the AIN-93M 
diet, whereas the two ER groups were fed by design 9.3 ± 1.7 
g/day of the modified AIN-93M-E diet. These intakes provided 
58.6 ± 10.5 and 34.8 ± 6.3 kcal/day, respectively, for CON 
and ER rats (Table 2) to achieve the intended 40% restriction 
of total energy intake. Food intake over the 12 wk was not 
different between VEH and BB rats within energy status 
groups. Energy density of the AIN-93M-E diet was lowered by 
reducing the primary carbohydrate source (corn starch) and 
replacing it with cellulose; hence the % of total kcal derived 
from bio-available carbohydrate was reduced to 60% vs. 76% 
in CON rats. Protein, fat, and important vitamin/mineral in-
takes were equivalent between the two groups (Table 2). Due to 
reduced total energy intake, the relative contribution of protein 
and fat to total energy intake was higher in ER groups 
than in CON groups (24% vs. 14% for protein and 16% vs. 9% 
for fat, respectively). Water intake was measured daily to 
verify propranolol dosing and did not vary significantly among 
groups (16–21.5 ml/day). Average propranolol dose delivered 
via water intake was 1.70 ± 0.2 and 1.77 ± 0.2 mg/day for 
CONBB and ERBB animals, respectively (P > 0.05 by un-
paired t-test).

ER produces large decreases in fat mass, smaller decreases in 
total body mineral content, and preserved lean mass. ERVEH 
and ERBB rats had lower body weights than CONVEH and 
CONBB rats by week 2 of treatment and through the end of 
the experiment (Fig. 1A). Over the 12-wk experimental period, ER 
rats’ body weight declined to 249 ± 12.63 g (−20%) in 
VEH-treated animals and to 254 ± 3.73 g (−18%) in the 
BB-treated group (P < 0.001 vs. week 0 within group), 
whereas CONBB rats gained body weight, weighing 347 ± 
6.44 g (+10.4%) at 12 wk (P < 0.01 vs. week 0). The smaller 
weight gain (24 g) in CONVEH rats over 12 wk was not 
significant (P < 0.2). Over 12 wk, control VEH and BB rats

| Table 2. Actual nutrient intake for pooled CON and ER groups |
|-------------------|-------------------|-------------------|
| Diet consumed, g  | 15.3 ± 2.7         | 9.3 ± 1.7         |
| Macronutrient     |                   |                   |
| Protein, g        | 2.1 ± 0.38        | 2.1 ± 0.39        |
| Carbohydrate, g   | 11.2 ± 1.97       | 5.2 ± 0.95†       |
| Fat, g            | 0.6 ± 0.11        | 0.6 ± 0.10        |
| Energy, kcal      | 58.6 ± 10.5       | 34.8 ± 6.3†       |
| Mineral and vitamin|                 |                   |
| Calcium, mg       | 76.5 ± 13.5       | 75.3 ± 13.7       |
| Potassium, mg     | 55.1 ± 9.7        | 53.9 ± 9.8        |
| Vitamin D, IU     | 15.3 ± 2.7        | 15.1 ± 2.7        |

ER, energy restriction. *There was no significant impact of β-adrenergic blockade on food intake in CON and ER groups; hence, all animals’ data within energy intake group were pooled for data in this table. †Significant difference vs. CON (P < 0.001).
the experimental period in all groups by week 4 (Table 3). ER alone (−91.34 mg/cm³), but not ER rats given BB (−62.68 mg/cm³), resulted in significantly greater reduction in total vBMD than in CONBB and CONVEH rats (−36.00 and −33.28 mg/ml, respectively). Cortical shell vBMD in CONBB and ERBB rats (+17 and +19 mg/cm³, respectively) tended to increase over 12 wk ($P < 0.07$), but this increment was not observed in VEH-treated rats (data not shown). Longitudinal vBMD measurements at midshaft tibia revealed a significant and similar (~2.0%) increment in cortical vBMD for all groups except ERVEH rats ($P < 0.001$), suggesting ER impairs age-related gains in cortical vBMD (Table 3).

β-Adrenergic blockade mitigates decreased cancellous bone formation and increased bone resorption during ER. The threefold decrease in %MS/BS observed with ER in vehicle-treated rats (vs. ad libitum-fed rats) was abolished by β-blockade (Fig. 3A). Although MAR was numerically reduced in both ER groups vs. ad libitum-fed controls, this reduction was significant only in ERVEH rats (Fig. 3B). These reductions in MAR and %MS/BS contributed to the 79% reduction in bone formation rate (Fig. 3C) in ERVEH rats; this reduction in bone formation rate with ER was significantly attenuated in ERBB rats. Reduced % osteoid surface (vs. CONVEH) was observed in both ER groups (ERVEH and ERBB rats; Fig. 3D). The twofold increase in % osteoclast surface observed in ERVEH rats was reversed in ERBB rats, which group mean was twofold lower than for ERVEH rats (Fig. 3E). Percent BV/TV in ERVEH rats was 29% lower than that in CONVEH rats; β-adrenergic blockade normalized %BV/TV in ER animals (Fig. 3F). No significant effects of β-adrenergic blockade on any histomorphometric parameter was observed in ad libitum-fed CON rats.

β-Adrenergic blockade mitigates decreased leptin localization on osteoblastic cells and increased marrow adiposity during ER. ER reduced the number of leptin-positive bone marrow adipocytes in vehicle-treated rats ninefold, but β-adrenergic blockade tended to abolish this reduction (Fig. 4, A–D;
In vivo pQCT measures of ER and/or BB effects on bone density and geometry at the proximal and mid-shaft tibia

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal Tibia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total vBMD, mg/cm³</td>
<td>699.2 ± 6.7</td>
<td>680.4 ± 9.4</td>
</tr>
<tr>
<td>Marrow area, cm²</td>
<td>7.9 ± 0.4</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>Total area, cm²</td>
<td>15.1 ± 0.5</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>Cortical area, cm²</td>
<td>5.0 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
</tbody>
</table>

| Tibia Diaphysis |        |         |
| Total vBMD, mg/cm³ | 699.2 ± 12 | 680.3 ± 12 | 666.2 ± 12 |
| Cortical area, cm² | 6.3 ± 0.3 | 6.6 ± 0.4 | 6.9 ± 0.5 |

Values are means ± SE. VEH, vehicle administration; BB, β-blocker administration. For all experimental groups, n = 10, except for CONVEH, in which n = 9. Significant difference vs. week 0 within group by 10.22.035 on October 30, 2017 http://jap.physiology.org/ Downloaded from J Appl Physiol ▪ doi:10.1152/japplphysiol.00187.2012 ▪ www.jappl.org

Table 3. In vivo pQCT measures of ER and/or BB effects on bone density and geometry at the proximal and mid-shaft tibia

The decline in the number of osteoblastic cells staining positive for leptin observed in ERVEH rats was not observed in ERBB rats (Fig. 4, E–H); these comparisons did not, however, achieve statistical significance (Table 4). No background staining was observed in negative control studies when primary antibody was omitted.

Adipocyte number in proximal femur marrow increased dramatically with ER in VEH-treated rats (172 ± 16/mm² vs. 92 ± 19/mm² in CONVEH rats) at week 12. This 87% increase in adipocyte counts observed with ER was abolished by β-adrenergic blockade (77 ± 16/mm²) (P < 0.05) (Fig. 4). There was no effect of β-adrenergic blockade on adipocyte number in ad libitum-fed controls.

β-Adrenergic blockade mitigated decline in serum leptin during ER, and the change in serum leptin level was positively correlated with bone formation rate. The reduction in serum leptin values vs. week 0 values was significantly less in ERBB (−1.14 ± 1.0 ng/ml) than in ERVEH (−5.31 ± 1.1 ng/ml) animals by week 12 (Fig. 5A). Changes in serum leptin over the experimental period were positively correlated with bone formation rate as measured in week 12 (r = 0.62; P < 0.0001) (Fig. 5B). Those rats exhibiting the greatest decline in serum leptin consistently had the lowest bone formation rate in distal femur cancellous bone. The absolute serum leptin level at week 12 was associated with bone formation rate (r = 0.54; P < 0.0001) (data not shown). Serum leptin level at week 12 normalized by fat mass (g) in ERBB rats (0.17 ± 0.01 ng/ml·g fat) tended to be greater than that of ERVEH (0.07 ± 0.02 ng/ml·g fat) (P < 0.07).

**DISCUSSION**

These experiments extend on previous work demonstrating interesting links between the sympathetic nervous system, regulation of serum leptin, and bone mass. To our knowledge, these are the first studies to demonstrate the impact of β-adrenergic blockade on alterations in cancellous bone mass, bone cell activity, marrow adiposity, and circulating leptin during prolonged ER. Our data demonstrate that blocking β-adrenergic signaling significantly mitigates reductions in metaphyseal cancellous vBMD and bone formation rate, abolishes the increased bone resorption, and attenuates the increase in marrow adiposity observed during ER in adult rats.

The propranolol dose used in this study (6 mg·kg⁻¹·day⁻¹) was chosen for its efficacy in preventing disuse-induced cancellous bone loss (4). Doses ranging from 0.1 to 5 mg/kg (intraperitoneal injection) of propranolol effectively prevent cancellous bone loss due to estrogen deficiency (after ovariectomy) (9). We recently demonstrated that the β-adrenergic blocker propranolol mitigates disuse-induced bone loss by modulating changes in both resorption and formation; propranolol treatments are nearly as effective as providing a leptin analog in attenuating bone loss in tail-suspended male rats (4). Importantly, in the present study, no significant differences in any outcome measures were observed with β-adrenergic blockade in energy-replete control rats. β-Adrenergic blockade exerts its bone protective effect in the context of ER and/or serum leptin deficiency, but not in the energy-balanced state. We note that the relatively high variability in the ad libitum-fed (CON) cancellous vBMD values likely contributed to our inability to detect a statistically significant decline in energy-replete ani-
formation rate (BFR; 2), demonstrate that osteoprotegerin (OPG) pathway. Previously published data by the receptor activator of nuclear factor (1).

In bone marrow/osteoblast co-culture; these changes are read-

RANKL expression by osteoblasts and formation of osteoclasts

ER rats was abolished by propranolol treatment. Regulation of

formation rate during ER.

reduction in %MS/BS rather than to changes in MAR, sug-

given propranolol (Fig. 2); the magnitude of change in CON-BB cancellous vBMD suggests there may be a negative biological effect of β-adrenergic blockade in energy-replete animals. However, we observed no significant decline in histomorphometric measure of bone volume (%BV/TV; Fig. 3F) in energy-replete rats given propranolol.

Attenuated loss of bone mass by β-adrenergic blockade may result from suppressed resorption or attenuated decrease of formation or both. Attenuated bone formation rate due to ER; all these decrements were successfully attenuated by β-adrenergic blockade. Reduced bone formation rate was more to reduction in %MS/BS rather than to changes in MAR, suggesting that declines in osteoblast recruitment rather than osteoblast activity per se were responsible for the reduced bone formation rate during ER. The dramatic increase of bone resorbing surface observed in ER rats was abolished by propranolol treatment. Regulation of osteoclast number by β-adrenergic blockade could be mediated by the receptor activator of nuclear factor κB-ligand (RANKL)/ osteoprotegerin (OPG) pathway. Previously published data demonstrate that β-adrenergic receptor agonist stimulates RANKL expression by osteoblasts and formation of osteoclasts in bone marrow/osteoblast co-culture; these changes are readily reversed by treatment with a β-adrenergic receptor antagon-

Fig. 3. Effect of VEH and BB during ER or CON in rats on proximal tibia mineral apposition rate (MAR; 3). % mineralizing surface (%MS/BS; 4), bone formation rate (BFR; 5), % osteoid surface (D), % osteoclast surface (E), and % bone volume/tissue volume (%BV/TV; F). Values are means ± SE. For all experiment groups, n = 10, except CONVEH and ERVEH in which n = 9. Bars sharing same letters are not significantly different.

In the present study, 12 wk of ER lowered body weight and fat mass but markedly increased marrow adipocyte numbers, which was associated with reduced bone formation. Increased marrow adiposity may reflect a shift in bone marrow stromal cell differentiation pathways away from osteoblastic lineages toward adipocytes, effectively reducing availability of mature osteoblasts capable of forming bone matrix (5, 23, 48). Recently published data demonstrate a similar dramatic increase in marrow adiposity with a similar (~30%) ER regimen, which also decreased bone mass and body fat in young growing mice (17). Interestingly, we observed in the present study that the dramatic increase in adipocyte counts observed with ER was abolished by β-adrenergic blockade (Fig. 4I). Given that body fat mass was not different between both ER groups (Fig. 1B), further studies investigating the mechanism by which marrow fat is altered, whereas fat stores external to bone are not affected by β-adrenergic signaling blockade, are merited.

Our ER protocol did not result in any loss of lean mass. Given that 8-wk-old rats are fully mature, the lack of change in lean mass in ER rats is quite intriguing. Previously published data indicate that moderate, adult-onset ER can attenuate sarcopenia or aging-induced muscle loss in a primate model (6, 15, 32–34). We have observed in separate studies higher lean mass in growing mice fed a 30% ER diet vs. that in ad libitum-fed animals (unpublished data). There is evidence that ER prevents the shift in muscle fiber-type distribution and delays cellular atrophy with aging (34), but further investigation is required to explain the underlying mechanisms for this phenomenon. Another possible explanation for lean mass loss in other protocols might be related to reduced absolute intake of protein; our rodent diets and feeding protocols were care-
Fig. 4. A–D: adipocytes positive for leptin expression (red staining) in the marrow space of proximal femur in CONVEH (A), CONBB (B), ERVEH (C), and ERBB (D). Note increased numbers of adipocytes in ERVEH samples but not in ERBB samples. C–H: osteoblastic cells positive for leptin expression in femur CONVEH (E), CONBB (F), ERVEH (G), and ERBB (H). Magnification is ×400. Red arrows point to leptin-positive regions. I: marrow adipocyte density (adipocyte no./mm²). Bars sharing same letters are not significantly different.
fully designed to provide the same absolute g protein intake for the 40% ER groups.

Although serum leptin in humans and rodents correlates mainly with fat mass, several other factors modulate circulating leptin levels. In both human and rodent models, stimulation of the sympathetic nervous system and activation of β-adrenergic receptors in particular decrease serum leptin levels (25, 50). The decline in circulating leptin with fasting is associated with activation of the β-adrenergic receptors and a decrease in insulin level (37, 47). Long-term restriction in rats (ranging from 50% to 80% of ad libitum energy intake) results in a substantial decrease in serum leptin and white adipose leptin mRNA (30). In the present study, β-adrenergic blockade had no apparent effect on the dramatic loss of body fat after 12 wk of moderate (~40%) ER, but it did mitigate the decline in serum leptin. Furthermore, changes in serum leptin over the experimental period were positively correlated with bone formation rate, which means the rats exhibiting the greatest decline in serum leptin had the lowest bone formation rate in distal femur cancellous bone. Propranolol-treated rats tended to have higher serum leptin per gram body fat as well, suggesting that leptin synthesis or release per unit adipocyte was greater when β-adrenergic signaling is blocked in ER rats. The exact mechanism for β-adrenergic blockade rescue of leptin synthesis or release is not clear. Stimulated adenylyl cyclase and subsequent PKA phosphorylation is thought to be the regulating mechanism of leptin release from adipose tissue. Elevated cyclic AMP inhibits leptin release by adipose tissue (20, 47).

We speculate that the positive effects of β-adrenergic blockade on mitigating bone loss with ER may derive from the synergistic effects of these two mechanism pathways, that is, one from the direct effect of β-adrenergic blockade on osteoblasts and the other from the indirect effect of β-adrenergic blockade on leptin release from adipocytes, resulting in rescue of suppressed leptin synthesis or leptin release.

White adipose tissue is the primary site of leptin synthesis and secretory regulation, but recent data indicate that leptin is also produced in skeletal muscle, fetal bone/cartilage, and human osteoblasts (12, 26, 40, 49). In the present study, leptin expression in bone marrow adipocytes and osteoblastic cells tended to be rescued in ER rats given propranolol; this group also exhibited mitigated reduction in serum leptin and cancellous bone mass. The hypothesis that β-adrenergic blockade increases leptin synthesis and/or release is consistent with the mitigated reduction in serum leptin we observed in propranolol-treated rats consuming less energy. But our data cannot address whether the leptin localizing to osteoblastic cells derives from nearby marrow adipocytes or from adipose tissue distant from bone or even derives from the osteoblastic cell itself. Further investigation on the effect of β-adrenergic block-

<table>
<thead>
<tr>
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<th>Osteoblastic Cells (P = 0.18)</th>
<th>Adipocytes (P = 0.12)</th>
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<tbody>
<tr>
<td>CONVEH</td>
<td>10.9 ± 2.9</td>
<td>31.2 ± 7.5</td>
</tr>
<tr>
<td>CONBB</td>
<td>9.3 ± 4.1</td>
<td>27.9 ± 11.6</td>
</tr>
<tr>
<td>ERVEH</td>
<td>1.2 ± 0.2</td>
<td>5.7 ± 1.0</td>
</tr>
<tr>
<td>ERBB</td>
<td>15.6 ± 7.4</td>
<td>28.9 ± 8.4</td>
</tr>
</tbody>
</table>

Values are means ± SE (no. of positive-stained cells/mm²). No significant differences were found among groups by ANOVA.

The impact of ER on the skeleton depends on both the duration of ER and the age of the animal. Previously published data indicate that in mice and rats the negative effect of ER on bone volume disappears if reduced energy intake continues past 1 yr of age. Furthermore, the higher bone mass observed in the energy-deprived animals (vs. ad libitum-fed controls) was maintained until the age of natural death during life-long ER (45). On the other hand, the impact of 40% ER in young or growing mice is generally negative, with decreases in cortical but not always cancellous bone mass in 3.5- to 6-mo-old mice, along with significant declines in lean mass, fat mass, serum leptin, and IGF-I (24). However, separate studies using much younger mice (3- to 12-wk-old) demonstrated decrements in both cortical and cancellous bone mass, along with significant increases in marrow adiposity (17). The present study, performed in female rats subjected to ER between 6 and 9 mo of age, demonstrates significant decrements in cancellous bone mass with minimal effects on mid-shaft cortical bone. It may be that age- or species-specific alterations in important endocrine factors or in bone marrow stromal cells differentiation

<table>
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<tr>
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<th>Bone formation rate (mm²/mm²/day)</th>
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<tbody>
<tr>
<td>WK0</td>
<td>0 ± 0.04</td>
</tr>
<tr>
<td>WK4</td>
<td>0 ± 0.04</td>
</tr>
<tr>
<td>WK8</td>
<td>0 ± 0.04</td>
</tr>
<tr>
<td>WK12</td>
<td>0 ± 0.04</td>
</tr>
</tbody>
</table>

$$p < 0.0001$$

**Fig. 5. A:** change (post-value in ng/ml) of serum leptin over 4 wk and over 12 wk of ER or CON in rats administered VEH or BB. *Significant difference vs. CONVEH at week 12 (P < 0.05). $Significant difference vs. CONVEH at week 12 (P < 0.05). &Significant difference vs. ERVEH at week 12 (P < 0.05). B:** correlation between change in serum leptin value (post-value-pre value in ng/ml) and bone formation rate (BFR) measured at 84 days for all animals.
capability could explain some of these bone compartment-specific differences.

One limitation of this study design is the altered macronutrient content of the ER diet. Following the approach of many rodent ER studies (13), energy content of the diet was achieved by reducing the carbohydrate content of the diet, replacing it with cellulose. We are unaware of any data suggesting that this magnitude reduction in CHO as % total kcal (60% vs. 76% in ad libitum-fed controls) has an independent effect on bone formation or resorption dynamics.

In summary, 40% ER over 12 wk in adult rats caused significant bone loss and marrow adiposity at the proximal tibia accompanied by reductions in circulating leptin. β-adrenergic blockade mitigated the ER-induced bone loss by attenuating declines in osteoblastic cell recruitment and activity and by abolishing increases in osteoclast surfaces. Propranolol treatment also mitigated the increase in marrow adiposity and reduced circulating leptin observed after ER. Reduction in serum circulating leptin and in leptin expression in bone marrow adipocytes and osteoblastic cells with ER was mitigated by β-adrenergic blockade. Taken together, these data suggest a strong role for β-adrenergic signaling from the SNS in the response of cancellous bone and marrow fat to reduced dietary energy intake.

Growing evidence suggests an association between use of β-blockade medications and reduced fracture risk in humans. Chronic use of β-blocker medications is associated with lower femoral neck fracture risk, probably by improving BMD, cortical bone geometry, and trabecular bone microarchitecture in postmenopausal women (8). Our results suggest that individuals using β-blocker medications to treat existing cardiovascular disease, who are often advised to lose weight as well, may benefit additionally if bone loss incurred during chronic ER is simultaneously mitigated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.B. conception and design of research; K.B. performed experiments; K.B. analyzed data; K.B. and S.A.B. interpreted results of experiments; K.B. and S.A.B. prepared figures; K.B. drafted manuscript; K.B. and S.A.B. edited and revised manuscript; K.B. and S.A.B. approved final version of manuscript.

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