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Role of Oestrogen Receptor Signaling in Skeletal Response to Leptin in Female *ob/ob* Mice

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Abstract

Leptin, critical in regulation of energy metabolism, is also important for normal bone growth, maturation and turnover. Compared to wild type (WT) mice, bone mass is lower in leptin-deficient *ob/ob* mice; osteopenia in growing *ob/ob* mice is due to decreased bone accrual, and is associated with reduced longitudinal bone growth, impaired cancellous bone maturation and increased marrow adipose tissue (MAT). However, leptin deficiency also results in gonadal dysfunction, disrupting production of gonadal hormones which regulate bone growth and turnover. The present study evaluated the role of increased oestrogen in mediating the effects of leptin on bone in *ob/ob* mice. Three-month-old female *ob/ob* mice were randomized into one of 3 groups: (1) *ob/ob* +vehicle (veh), (2) *ob/ob*+leptin (leptin), or (3) *ob/ob*+leptin and the potent oestrogen receptor antagonist ICI 182,780 (leptin+ICI). Age-matched WT mice received vehicle. Leptin (40 μg/mouse, daily) and ICI (10 μg/mouse, 2x/w) were administered by sc injection for 1 month and bone analyzed by x-ray absorptiometry, microcomputed tomography, and static and dynamic histomorphometry. Uterine weight did not differ between *ob/ob* mice and *ob/ob* mice receiving leptin+ICI, indicating that ICI successfully blocked the uterine response to leptin-induced increases in oestrogen levels. Compared to leptin-treated *ob/ob* mice, *ob/ob* mice receiving leptin +ICI had lower uterine weight, did not differ in weight loss, MAT, or bone formation rate, and had higher longitudinal bone growth rate and cancellous bone volume fraction. We conclude that increased oestrogen signaling following leptin treatment is dispensable for the positive actions of leptin on bone and may attenuate leptin-induced bone growth.

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Keywords

Oestrogen; leptin; bone formation and resorption; skeletal biology; marrow adipose tissue

Introduction

Leptin plays a role in regulating the rate of bone elongation, maturation of primary spongiosa, cortical and cancellous bone accrual, and cancellous bone turnover (Turner, et al. 2013). Leptin-deficient *ob/ob* mice and leptin receptor-deficient *db/db* mice have bone- and bone compartment-specific alterations in bone microarchitecture, reduced bone length, mass and density, and reduced bone quality (Jing, et al. 2016; Williams, et al. 2011). The growth plates of *ob/ob* mice are abnormal due, in part, to poorly organized collagen fibril arrangement (Kishida, et al. 2005). The pathological manifestations in growth plate are associated with decreased type X collagen expression, increased chondrocyte apoptosis and premature mineralization (Kishida et al. 2005), suggesting that leptin modulates events associated with terminal differentiation of chondrocytes. Importantly, the defects in growth plate and bone microarchitecture are largely reversed in growing *ob/ob* mice following administration of leptin (Kishida et al. 2005; Kume, et al. 2002; Maor, et al. 2002).

Osteoblast and osteoclast differentiation and/or function are also impaired in leptin signaling-deficient *ob/ob* and *db/db* mice (Turner et al. 2013). The lower bone formation generally reported in these mice is primarily due to reduced osteoblast number, although reduced osteoblast activity has also been noted. The dramatic increase in bone marrow adipose tissue (MAT) in long bones of leptin signaling-deficient mice suggests that leptin regulates bone marrow mesenchymal stem cell lineage decision; in the absence of leptin there is a net increase in marrow adipocytes and a decrease in osteoblasts. In contrast to a reduction in osteoblast perimeter, osteoclast perimeter is either normal or increased in *ob/ob* and *db/db* mice (Turner et al. 2013). However, bone resorption is reduced, implicating impaired osteoclast activity. The osteoclast defect in these mice contributes to growth plate abnormalities and development of mild osteopetrosis: the pathological retention of calcified cartilage into adulthood in bone in *ob/ob* and *db/db* mice likely contributes to the reduced bone quality noted in these animals (Jing et al. 2016).

Although absence of leptin signaling is ultimately responsible for the skeletal abnormalities in *ob/ob* and *db/db* mice, leptin is a pleiotropic hormone, impacting many physiological systems, including energy partitioning, thermogenesis, immune regulation, and gonadal function, each of which could independently influence bone metabolism (Mantzoros, et al. 2011). Leptin is required for the release of gonadotropin-releasing hormone (GnRH) from the pituitary and, as a consequence, female *ob/ob* mice have greatly reduced oestrogen levels and exhibit low uterine weight (Barash, et al. 1996). GnRH-dependent oestrogen synthesis and release play an important role in regulating bone elongation, accrual and turnover, and growing oestrogen-deficient rodents exhibit skeletal abnormalities, including accelerated bone growth, increased bone turnover and cancellous bone loss (Burguera, et al. 2001; Turner, et al. 1994).

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As mentioned, administration of leptin to *ob/ob* mice reverses many, if not all, of the skeletal abnormalities associated with leptin deficiency (Bartell, et al. 2011; Hamrick, et al. 2005; Iwaniec, et al. 2007; Kishida et al. 2005). However, leptin administration to *ob/ob* mice also restores gonadal function (Barash et al. 1996) and in WT mice accelerates the onset of puberty (Ahima, et al. 1997). Thus, it is possible that oestrogen deficiency contributes to the skeletal phenotype observed in leptin signaling-deficient rodents and restoration of oestrogen receptor signaling following leptin administration may influence the physiological response of *ob/ob* mice to leptin. This possibility is consistent with the results of a recent leptin dose response study conducted in *ob/ob* mice (Philbrick, et al. 2017). Specifically, a pronounced dose-dependent stimulatory effect of leptin on bone accrual plateaued at a dose rate that increased uterine weight (an index of oestrogen levels). Therefore, the current study was designed to determine the precise role of oestrogen signaling in the skeletal response to leptin. This was accomplished by administering leptin sc to *ob/ob* mice in the presence or absence of the potent oestrogen receptor antagonist ICI 182,780 (ICI) (Wakeling and Bowler 1992). Treatment with ICI replicates the uterine and skeletal responses to ovariectomy (ovx) in rodents, and thus its administration to leptin treated *ob/ob* mice should prevent actions mediated through increased oestrogen levels (Sibonga, et al. 1998).

Materials and Methods

Experimental design

One-month-old *ob/ob* and WT mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained until 3 months of age. The *ob/ob* mice were then randomized by weight into onto one of 3 treatment groups: (1) *ob/ob*+vehicle (veh) (n=5), (2) *ob/ob*+leptin (leptin) (n=8), or (3) *ob/ob*+leptin+ICI (leptin+ICI) (n=8). A group of WT mice (WT) (n=9) received vehicle. Recombinant mouse leptin (CYT-351, Prospec Bio, Rehovot, Israel) was administered once daily by sc injection at a dose of 40 μg/mouse/d in 0.1 ml phosphate buffered saline (vehicle). ICI was administered 2x/w by sc injection at a dose of 10 μg/mouse in 0.1 ml extra virgin olive oil. Fluorochromes were administered at 9 days (declomycin, 15 mg/kg; Sigma Chemical, St Louis, MO), 4 days (calcein, 15 mg/kg; Sigma Chemical, St Louis, MO) and 1 day (calcein) prior to necropsy to label mineralizing bone. Food (Teklad 8604, Harlen Laboratories, Indianapolis, IN) and water were provided *ad libitum* to all animals. Body weight was recorded daily. Food intake was also recorded daily, except for days 12-14, 19-20, and 26-28 when it was averaged over a 2-3 day interval. The mice were maintained at room temperature (22°C) and singly housed on a 12 h light:12 h dark cycle for the duration of study. For tissue collection, mice were anesthetized using isoflurane and killed by cardiac exsanguination followed by decapitation. Uteri and abdominal white adipose tissue (WAT) were excised and weighed. Femora and 5^{th} lumbar vertebrae were removed, fixed for 24h in 10% buffered formalin, and stored in 70% ethanol for dual energy absorptiometry (DXA), microcomputed tomography (μCT), and histomorphometric analyses. The experimental protocol was approved by the Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

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Densitometry—Total femur bone mineral content (BMC, g), bone area (cm^2), and bone mineral density (BMD, g/cm^2) were measured using DXA (Piximus 2, Lunar Corporation, Madison, WI).

Micro-computed tomography—Microcomputed tomography (μCT) was used for nondestructive 3-dimensional evaluation of bone volume and architecture. Femora and lumbar vertebrae were scanned in 70% ethanol using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 12 × 12 × 12 μm (55 kV_p x-ray voltage, 145 μA intensity, and 200 ms integration time). Filtering parameters sigma and support were set to 0.8 and 1, respectively. Voxels having a threshold ≥ 245 (0 – 1000) were used to distinguish bone from non-bone. Total femur mineralized tissue volume (cancellous + cortical bone) was evaluated. This was followed by site-specific evaluation of cortical bone in the midshaft femur and cancellous bone in the distal femur metaphysis and epiphysis. For the femoral midshaft, 20 slices (0.24 mm) of bone were evaluated and total cross-sectional tissue volume (cortical and marrow volume, mm^3), cortical volume (mm^3), marrow volume (mm^3), cortical thickness (μm) and polar moment of inertia (mm^4, an index of bone strength in torsion) were measured. For the femoral metaphysis, 42 slices (0.50 mm) of cancellous bone were measured, 45 slices (0.54 mm) proximal to the growth plate/metaphysis boundary. For the femoral epiphysis, the entire cancellous compartment (33 ± 1 slices) between the distal epiphyseal growth plate and distal femoral cortical shell was measured. μCT analysis of lumbar vertebrae was performed on cancellous bone within the vertebral body, between the cranial and caudal growth plates. Irregular manual contouring a few voxels interior to the endocortical surface was used to delineate cancellous from cortical bone. Direct cancellous bone measurements in the distal femur metaphysis and epiphysis and in lumbar vertebra included cancellous bone volume fraction (bone volume/tissue volume, %), trabecular thickness (μm), trabecular number (mm^{-1}), and trabecular separation (μm).

Histomorphometry

Distal femora were dehydrated in a graded series of ethanol and xylene, and embedded undecalcified in modified methyl methacrylate as described (Iwaniec, et al. 2008). Coronal sections (4 μm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to gel coated slides. One section per animal was stained for tartrate resistant acid phosphatase and counter stained with toluidine blue and used for cell-based measurements. A second section was left unstained for dynamic histomorphometry. Histomorphometric data were collected with a 20× objective using the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA). The sampling site for the distal femoral metaphysis was located 0.25-1.25 mm proximal to the growth plate and 0.1 mm from cortical bone.

Cell-based measurements included osteoblast perimeter (osteoblast perimeter/bone perimeter, %), osteoclast perimeter (osteoclast perimeter/bone perimeter, %), marrow adiposity (adipocyte area/tissue area, %), adipocyte density (number of adipocytes/tissue area, $\#/mm^2$) and adipocyte size (μm^2). Osteoblasts, osteoclasts, and adipocytes were identified as previously described (Iwaniec, et al. 2016). Fluorochrome-based measurements of bone formation included mineralizing perimeter (mineralizing perimeter/bone perimeter:

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cancellous bone perimeter covered with double plus half single label normalized to bone perimeter, %), mineral apposition rate (the mean distance between two fluorochrome markers that comprise each double label divided by the 3-d interlabel interval, μm/d), and bone formation rate adjusted for bone perimeter (bone formation rate/bone perimeter: calculated by multiplying mineralizing perimeter by mineral apposition rate normalized to bone perimeter, $\mu m^2/\mu m/y$). In addition, longitudinal growth rate was determined as the mean distance from the declomycin label to the mineralizing growth plate cartilage divided by the 9-d interval from label administration to sacrifice. All bone histomorphometric data are reported using standard 2-dimensional nomenclature (Dempster, et al. 2013).

Statistics

Longitudinal data on body weight and food intake were analyzed using multivariate linear regression models with separate linear (body weight) or constant (food intake) time trends across groups. Candidate covariance models included independence, compound symmetric with and without equal variance across time and group, autoregressive of order 1, and moving average of order 1. Model selection was based on the Bayesian information criterion.

The principal goal of the analyses of tissues harvested at necropsy was to determine whether combination treatment (leptin+ICI) differed from leptin treatment. To accomplish this goal, mean responses of individual variables were compared for WT, vehicle, leptin, and leptin +ICI mice using one-way analysis of variance, with Dunnett's used to adjust for making multiple comparisons to the leptin+ICI group. The Kruskal-Wallis nonparametric test was used when only the normality assumption was violated, and a modified F-test was used when the assumption of equal variance was violated (Welch 1951); in these cases, the Wilcoxon-Mann-Whitney test or Welch's two-sample t-test was used for pairwise comparisons and the Holm procedure was used to adjust for multiple comparisons (Holm 1979). The required conditions for valid use of Gaussian analysis of variance models were assessed using Levene's test for homogeneity of variance, plots of residuals versus fitted values, normal quantile plots, and the Anderson-Darling test of normality. Data analysis was performed using R version 3.3.2 (Team 2015). Differences were considered significant at $P \leq 0.05$. All data are expressed as mean ± standard error.

Results

The effects of leptin deficiency and treatment with leptin and leptin+ICI on body weight and food consumption in *ob/ob* mice are shown in Figure 1A and 1B, respectively. Vehicle treated *ob/ob* mice weighed more and consumed more food than WT mice throughout the 4-week study. A progressive decrease in weight was observed in leptin treated mice but weight remained higher than in WT mice for the duration of treatment. Food consumption decreased in leptin treated and leptin+ICI treated *ob/ob* mice during the initial week of treatment. Mice treated with leptin+ICI did not differ from leptin treated mice in body weight or food intake.

The effects of treatment on abdominal WAT weight and uterine weight in *ob/ob* mice are shown in Figure 2A and B, respectively. WAT weight in leptin+ICI treated mice was higher

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than in WT mice, lower than in vehicle treated mice but did not differ from leptin treated mice. In contrast, uterine weight in leptin+ICI treated mice was lower than in WT mice, did not differ from vehicle treated mice and was lower than in leptin treated mice.

The effects of treatment on femur DXA measurements, and microcomputed tomography measurements of cortical bone architecture in the midshaft femur, and cancellous bone architecture in the distal femur metaphysis and epiphysis in *ob/ob* mice are shown in Table 1. Leptin+ICI treated mice did not differ from WT mice in total femur BMC, bone area, BMD, or bone volume. Femur length and cortical thickness were lower in leptin+ICI treated mice compared to WT mice while cross sectional bone volume, marrow volume and polar moment of inertia were higher. Bone volume fraction in distal femur metaphysis and epiphysis was higher and trabecular spacing lower in the leptin+ICI treated mice compared to WT mice and this was due to higher trabecular number and thickness in the metaphysis and higher trabecular number in the epiphysis. Leptin+ICI treated mice had longer femurs and higher BMC and BMD than vehicle treated mice. Similarly, cortical volume, thickness and polar moment of inertia were higher in the leptin+ICI treated mice. Bone volume fraction did not differ between leptin+ICI treated and vehicle treated mice in the metaphysis but was higher in the leptin+ICI treated mice in the epiphysis. The latter was associated with lower trabecular number and higher trabecular thickness in the leptin+ICI treated mice. Leptin+ICI treated mice did not differ from leptin treated mice in femur length, femur bone volume or cortical bone architecture. However, the two groups differed in cancellous bone architecture; leptin+ICI treated mice had higher cancellous bone volume fraction in the metaphysis which was associated with higher trabecular number, no change in trabecular thickness and lower trabecular spacing.

The effects of treatment on cancellous bone microarchitecture in 5th lumbar vertebra of *ob/ob* mice are shown in Table 2. Leptin+ICI treated mice had higher bone volume fraction than WT mice; the higher cancellous bone volume fraction was associated with higher trabecular number, no difference in trabecular thickness and lower trabecular spacing. Leptin +ICI treated mice had lower bone volume fraction than vehicle treated mice. However, significant differences were not detected between the two groups in any of the architectural endpoints evaluated. Bone volume fraction, connectivity density, trabecular number and trabecular spacing did not differ between leptin+ICI and leptin treated mice.

The effects of treatment on dynamic bone histomorphometry in distal femur metaphysis of *ob/ob* mice are shown in Figure 3A-D. Leptin+ICI treated mice had higher longitudinal growth rate and mineralizing perimeter than WT mice but significant differences between the two groups were not detected in mineral apposition rate or bone formation rate. Leptin +ICI treated mice had higher values for each of these endpoints than vehicle treated mice. Leptin+ICI treated mice had higher longitudinal growth rate than leptin treated mice but mineralizing bone perimeter, mineral apposition rate and bone formation rate did not differ between the two groups.

The effects of treatment on osteoblast perimeter, osteoclast perimeter and MAT in the distal femur metaphysis *of ob/ob* mice are shown in Figure 3E-I. Leptin+ICI treated mice had higher osteoblast perimeter, MAT area, adipocyte density and adipocyte size than WT mice

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but osteoclast perimeter did not differ between the two groups. Leptin+ICI treated mice had higher osteoblast perimeter and lower osteoclast perimeter, MAT area, and adipocyte density than vehicle-treated mice. Leptin+ICI and leptin treated mice did not differ in any of the cellular endpoints evaluated. The differences among groups in fluorochrome label and MAT can be readily appreciated in Figure 3J-M.

Discussion

Oestrogens regulate bone growth and turnover and are essential for the sexual dimorphism of the skeleton (Turner et al. 1994). In growing rodents, endogenous oestrogens promote termination of longitudinal bone growth and have bone- and bone compartment-specific effects on bone acquisition and turnover balance (Turner, et al. 1992; Turner, et al. 1993). ICI is a fluorinated steroid which binds to oestrogen receptors with high affinity and lowers oestrogen receptor levels by enhancing their proteosomal degradation (Dauvois, et al. 1993; Wakeling and Bowler 1992). When administered to growing ovary-intact rodents ICI, similar to ovx, results in uterine atrophy, increased longitudinal bone growth and cancellous bone loss (Sibonga et al. 1998).

Leptin increases longitudinal bone growth in *ob/ob* mice (Kishida et al. 2005). The further increase in growth rate in *ob/ob* mice treated with leptin+ICI provides evidence that the concurrent increase in oestrogen levels during leptin treatment antagonizes the growth promoting actions of leptin (Turner et al. 1994). Cancellous bone volume fraction was also higher in the distal femur metaphysis of ICI treated *ob/ob* mice compared to leptin treated *ob/ob* mice. This finding was not necessarily anticipated because oestrogen deficiency typically results in decreased cancellous bone volume fraction due to reduced trabecular number. However, this positive skeletal effect of ICI on cancellous bone volume fraction was associated with the noted accelerated longitudinal bone growth. Concurrent increases in longitudinal growth and bone formation rates is a plausible explanation for the higher cancellous bone volume fraction in leptin+ICI treated mice. This interpretation is consistent with prior studies demonstrating that sc administration of leptin reduces bone loss in growing ovx rats by attenuating the decrease in trabecular number (Burguera et al. 2001).

Ovx results in a reduction in cortical thickness in mice that is preventable by oestrogen replacement (Hawse, et al. 2014). In the present study, cortical thickness was increased in hypogonadal *ob/ob* mice following leptin treatment when compared to untreated *ob/ob* mice but cortical thickness was not restored to WT levels. Prior studies reported reduced endocortical bone formation in WT mice following adoptive transfer of bone marrow from leptin receptor deficient *db/db* mice, and increased endocortical bone formation in *ob/ob* mice following leptin administration (Turner et al. 2013). Thus, the reduced cortical thickness in femurs of *ob/ob* mice could result from oestrogen deficiency, leptin deficiency or combined deficiencies of the two hormones. The absence of an effect of ICI in leptin treated mice indicates that the resulting increase in cortical thickness does not require oestrogen signaling. However, increasing leptin levels while reducing the magnitude did not fully prevent bone loss in ovx rats (Burguera et al. 2001) Taken together, these observations suggest that leptin and oestrogen have distinct as well as complementary effects on the skeleton and that both hormones may be required for optimal cortical thickness.

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Oestrogens play an important role in energy balance. Rodents typically exhibit excess weight gain following ovx due in part to hyperphagia (Clark and Tarttelin 1982). Pair-feeding ovx rodents to ovary-intact controls attenuates but does not completely prevent excess weight gain, suggesting that ovarian hormones also increase energy expenditure (Jiang, et al. 2008). The effects of oestrogen on energy balance are believed to occur through oestrogen receptor-mediated signaling within the hypothalamus (Frank, et al. 2014). ICI delivered into peripheral circulation may cross the blood brain barrier and localize in the hypothalamus (Alfinito, et al. 2008; Howell, et al. 2000). As such, sc administered ICI could antagonize oestrogen receptor-mediated pathways involved in energy metabolism in the central nervous system. In the present study, ICI did not alter the effects of leptin on food consumption or slow weight loss in *ob/ob* mice. Additionally, hypothalamic leptin gene therapy was shown to be effective in blocking the increase in appetite in rats following ovx (Ng, et al. 2010; Torto, et al. 2006). These findings suggest that a leptin-induced increase in oestrogen levels is not required for leptin to suppress appetite. However, the dose rate of ICI used in the present study, although sufficient to block oestrogen receptor-mediated actions on uterus and bone (Sibonga et al. 1998), was lower than the concentrations shown to induce hyperphagia and decrease energy expenditure in normal rats (Alfinito et al. 2008). It is therefore possible that higher doses of ICI are required to block the inhibitory actions of leptin-induced oestrogen on appetite.

ob/ob mice develop morbid obesity. In contrast to adult WT mice, which were nearly weight stable throughout the one-month duration of our study, *ob/ob* mice gained weight. As anticipated, leptin treatment resulted in weight loss, which was due in part to lower abdominal WAT weight. We did not measure individual fat depots or lean mass but leptin treatment has been shown to result in a preferential reduction in total fat mass with minimal reduction in water and lean body mass (Rafael and Herling 2000). Total body weight and abdominal WAT weight, although lower than in untreated *ob/ob* mice, were still much higher at study termination in leptin treated *ob/ob* mice than in WT mice. Similar to appetite, the absence of an effect of co-treatment with leptin and ICI on weight compared to leptin alone suggests that oestrogen receptor signaling is largely dispensable for the leptin-mediated reduction in WAT and body weight. This conclusion is concordant with studies demonstrating the efficacy of leptin in preventing weight gain in ovx mice and rats (da Silva, et al. 2014; Torto et al. 2006).

Adipose tissue produces a wide array of peptide hormones and cytokines, collectively referred to as adipokines (Ronti, et al. 2006). Additionally, adipose tissue expresses aromatase activity and production of estrogens by WAT may contribute to the higher oestrogen levels associated with obesity (Cleland, et al. 1983, 1985; Liu, et al. 2013; Magoffin, et al. 1999). Uterine weight, a sensitive index of oestrogen level, was positively associated with WAT weight in *ob/ob* mice (Turner, et al. 2014). Leptin treatment increased uterine weight in *ob/ob* mice (Turner et al. 2014) and this response was blocked by ICI, indicating that ICI treatment was effective in blocking oestrogen signaling, regardless of the tissue origin of the hormone.

In spite of drastic differences in abdominal WAT between calorically restricted mice (decreased) and mice with loss of function mutations in the gene for leptin or the gene for

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leptin receptor (increased), both conditions result in increased MAT (Bartell et al. 2011; Turner and Iwaniec 2011), a finding implicating leptin as an important negative regulator of MAT. This conclusion is supported by studies demonstrating that increasing leptin levels by sc administration of the hormone, direct delivery of the hormone into the hypothalamus, or by hypothalamic leptin gene therapy reduces MAT in *ob/ob* mice (Bartell et al. 2011; Turner, et al. 2015). However, ovx rats and normal mice fed high fat diet have elevated MAT in spite of elevated leptin levels, and increasing hypothalamic leptin levels does not decrease MAT in normal and ovx rats (Iwaniec, et al. 2011; Jackson, et al. 2011; Martin and Zissimos 1991). This paradox is resolved if, similar to appetite and weight gain, the inhibitory effects of high levels of leptin on MAT are impaired by development of leptin resistance (Sainz, et al. 2015). In the present study, the reduction in MAT by sc administration of leptin to leptin-deficient mice was not prevented by co-administration with ICI. Taken together, these findings suggest oestrogen receptor signaling is not required for leptin to reduce MAT levels in *ob/ob* mice while leptin resistance may facilitate the increase in MAT associated with ovx.

Accurately modeling the diurnal fluctuations observed in serum leptin levels is challenging (Arble, et al. 2011). In normal mice, leptin levels display a diurnal rhythm with a nadir in mid-morning and a nocturnal peak (Ahren 2000). In the present study, we administered leptin to leptin-deficient *ob/ob* mice by daily sc injection. This route of administration would be expected to result in much greater extremes in maximum and minimum levels of the hormone than occur physiologically. Nevertheless, once daily sc leptin treatment quickly decreased food intake to values similar to WT mice and maintained lower food intake through the remainder of study.

Bone balance is highly sensitive to changes in energy availability (Iwaniec and Turner 2016). Caloric restriction in adult rats, resulting in only 5% weight loss, had dramatic negative effects on bone metabolism, including reduced bone formation, increased osteoclast perimeter and reduced cancellous bone volume fraction (Turner and Iwaniec 2011). In contrast, increases in leptin levels in the hypothalamus of normal rats reduced food intake and body weight without inducing bone loss (Turner et al. 2015). However, a combination of caloric restriction (30%) and sc leptin treatment, while reducing MAT accumulation, did not prevent the detrimental skeletal changes associated with suppression of normal weight gain in rapidly growing mice (Devlin, et al. 2016). These findings suggest that methods that improve leptin signaling have the potential to preserve bone mass during rapid weight loss in adults but are unlikely to compensate for inadequate energy availability during growth.

Adoptive transfer of leptin receptor-deficient *db/db* bone marrow cells into WT mice recapitulated the low bone formation observed in *db/db* mice without increasing food intake (Turner et al. 2013). Additionally, sc infusion of leptin revealed that the stimulatory effects of leptin on bone formation occur at leptin levels that have minimal effects on energy metabolism (Philbrick et al. 2017). These findings provide strong evidence that leptin acts peripherally to stimulate bone formation. However, the putative target cells in bone and precise mechanisms mediating the bone anabolic effects of leptin in *ob/ob* mice have not been identified.

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It is likely that some of the profound abnormalities in bone metabolism in leptin-deficient *ob/ob* mice occur as a result of comorbidities and hormonal changes. Comorbidities include hypogonadism, hyperphagia, impaired thermoregulation, and hyperglycemia. Alterations in bone regulating hormones and growth factors include increased corticosteroid levels, and low levels of sex steroids, growth hormone and IGF1 (Ozata, et al. 1999; Saito and Bray 1983; Segev, et al. 2007; Turner et al. 2014). Additionally, hyperparathyroidism is associated with leptin deficiency in humans (Ozata et al. 1999). In the present study, we demonstrate that ovarian hormones play a limited role in mediating the skeletal actions of leptin. In prior studies, we showed that hyperphagia, hyperglycemia and impaired thermoregulation, typically observed in *ob/ob* mice, actually attenuate skeletal abnormalities in these animals (Turner et al. 2014). Taken together, these findings indicate that leptin plays an important role in regulating bone growth, maturation and turnover but further research is required to understand the full range of actions by the hormone.

In summary, the profound abnormalities in metabolism in *ob/ob* mice, including hyperphagia and morbid obesity were attenuated or reversed following combination treatment with leptin and the oestrogen receptor antagonist ICI. Similarly, leptin+ICI re-established compensatory increases in bone growth and near normal bone turnover, without restoring normal uterine weight. The increase in osteoblast-lined bone perimeter and bone formation in leptin+ICI treated *ob/ob* mice was accompanied by a reduction in MAT, suggesting that treatment promoted the differentiation of stromal cells to osteoblasts at the expense of adipocytes. Finally, the limited differences in skeletal endpoints between *ob/ob* mice treated with leptin compared to *ob/ob* mice treated with leptin+ICI indicate that oestrogen receptor signaling is dispensable for the positive actions of leptin on bone and that oestrogen may attenuate leptin-induced bone growth.

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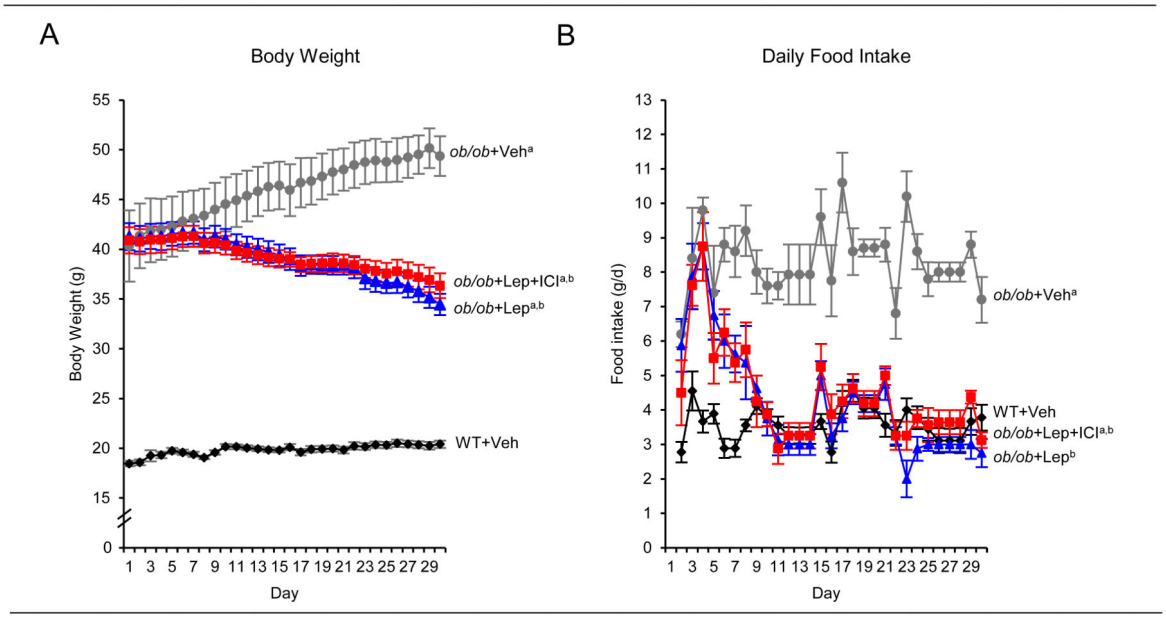

Figure 1.

Effects of 1 month of daily sc leptin injection (40 μg/mouse/d) in the presence or absence of the potent oestrogen receptor antagonist ICI 182,780 (ICI) on body weight (A) and food intake (B) in female *ob/ob* mice. Data are mean ± SE, n=5-9/group. [a]Different from WT mice, P<0.05. [b]Different from vehicle-treated *ob/ob* mice, P<0.05.

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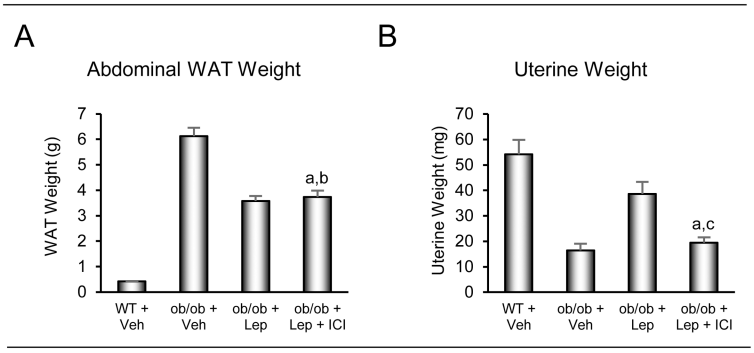

Figure 2.

Effects of 1 month of daily sc leptin injection (40 μg/mouse/d) in the presence or absence of the potent oestrogen receptor antagonist ICI 182,780 (ICI) on abdominal white adipose tissue (WAT) weight (A) and uterine weight (B) in female *ob/ob* mice. Data are mean ± SE, n=5-9/group. [a]Leptin+ICI treated *ob/ob* mice different from vehicle treated WT mice, $P<0.05$; [b]Leptin+ICI treated *ob/ob* mice different from vehicle-treated *ob/ob* mice, $P<0.05$; [c]Leptin+ICI treated *ob/ob* mice different from leptin-treated *ob/ob* mice, $P<0.05$.

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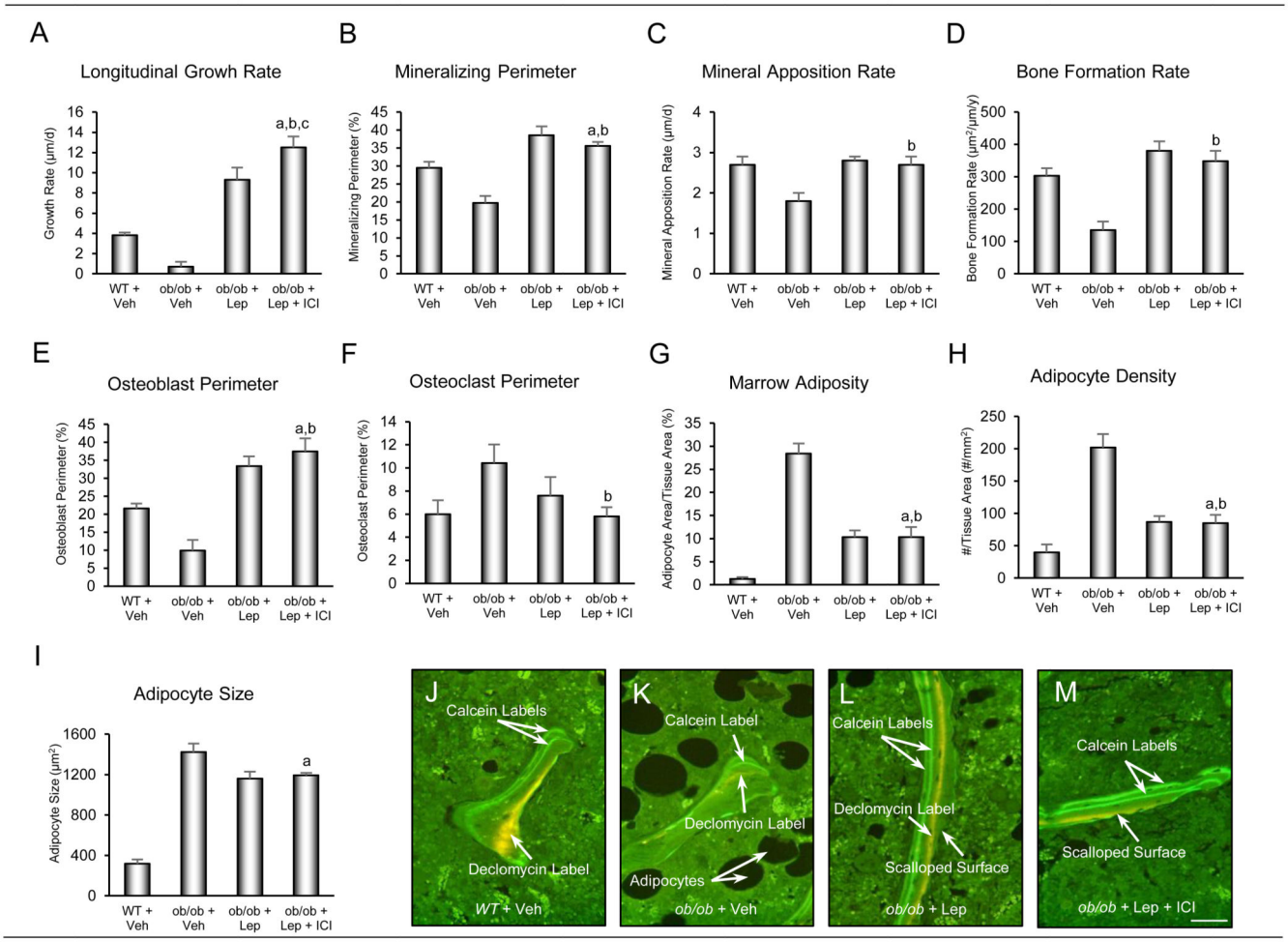

Figure 3.

Effects of 1 month of daily sc leptin injection (40 μg/mouse/d) in the presence or absence of the potent oestrogen receptor antagonist ICI 182,780 (ICI) on longitudinal bone growth rate (A), mineralizing perimeter (B), mineral apposition rate (C), bone formation rate (D), osteoblast perimeter (E), osteoclast perimeter (F), marrow adiposity (G), adipocyte density (H) and adipocyte size (I) in distal femur metaphysis in female *ob/ob* mice. Representative photomicrographs illustrating differences in fluorochrome labeling in WT mice treated with vehicle (J), *ob/ob* mice treated with vehicle (K), *ob/ob* mice treated with leptin (L) and *ob/ob* mice treated leptin and ICI (M). Data are mean ± SE, n=5-9/group. [a]Leptin+ICI treated *ob/ob* mice different from vehicle treated WT mice, P<0.05; [b]Leptin+ICI treated *ob/ob* mice different from vehicle-treated *ob/ob* mice, P<0.05; [c]Leptin+ICI treated *ob/ob* mice different from leptin-treated *ob/ob* mice, P<0.05. Scale bar, 50 μm.

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Table 1

Effects of 1 month of daily sc leptin injection (40 μg/mouse/d) in the presence or absence of the potent antiestrogen ICI 182,780 (ICI) on femur bone mineral content and density and on cortical bone architecture in midshaft femur, and cancellous bone architecture in distal femur metaphysis and epiphysis in female *ob/ob* mice.

End Point	WT + Veh	*ob/ob* + Veh	*ob/ob* + Lep	*ob/ob* + Lep + ICI	ANOVA P Value
Dual Energy X-ray Absorptiometry					
Total Femur					
BMC (g)	0.019 ± 0.000	0.016 ± 0.001	0.017 ± 0.000	0.019 ± 0.001^{b}	0.0011
Bone area (cm^2)	0.40 ± 0.01	0.38 ± 0.01	0.40 ± 0.01	0.41 ± 0.01	0.1000
BMD (g/cm^2)	0.047 ± 0.001	0.041 ± 0.001	0.044 ± 0.000	0.046 ± 0.001^{b}	0.0010
Microcomputed Tomography					
Total Femur					
Length (mm)	15.2 ± 0.1	13.5 ± 0.2	14.0 ± 0.1	14.0 ± 0.1^{a,b}	0.0000
Bone volume (mm^3)	16.2 ± 0.2	13.5 ± 0.5	15.1 ± 0.3	15.6 ± 0.3^{b}	0.0000
Mishaft Femur (cortical bone)					
Cross-sectional volume (mm^3)	0.34 ± 0.00	0.38 ± 0.00	0.38 ± 0.01	0.40 ± 0.01^{a}	0.0000
Cortical volume (mm^3)	0.16 ± 0.00	0.14 ± 0.00	0.16 ± 0.00	0.16 ± 0.00^{b}	0.0002
Marrow volume (mm^3)	0.19 ± 0.00	0.24 ± 0.01	0.23 ± 0.01	0.24 ± 0.01^{a}	0.0000
Cortical thickness (μm)	183 ± 3	151 ± 3	167 ± 3	164 ± 2^{a,b}	0.0000
Polar moment of inertia (mm^4)	0.29 ± 0.01	0.30 ± 0.01	0.34 ± 0.01	0.35 ± 0.01^{a,b}	0.0004
Distal Femur Metaphysis (cancellous bone)					
Bone volume/tissue volume (%)	4.4 ± 0.4	6.6 ± 1.1	6.2 ± 0.5	7.9 ± 0.3^{a,c}	0.0003
Trabecular number (mm^{-1})	3.8 ± 0.1	4.0 ± 0.2	3.8 ± 0.1	4.1 ± 0.04^{a,c}	0.0046
Trabecular thickness (μm)	40 ± 1	42 ± 2	44 ± 1	43 ± 1^{a}	0.0431
Trabecular spacing (μm)	273 ± 5	261 ± 15	275 ± 4	257 ± 4^{a,c}	0.0293
Distal Femur Epiphysis (cancellous bone)					
Bone volume/tissue volume (%)	25.9 ± 0.5	26.5 ± 0.3	29.9 ± 0.8	31.4 ± 0.6^{a,b}	0.0001
Trabecular number (mm^{-1})	5.1 ± 0.2	7.4 ± 0.4	6.3 ± 0.2	6.6 ± 0.2^{a,b}	0.0000

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End Point	WT + Veh	*ob/ob* + Veh	*ob/ob* + Lep	*ob/ob* + Lep + ICI	ANOVA P Value
Trabecular thickness (μm)	61 ± 1	50 ± 0	59 ± 1	60 ± 1[b]	0.0000
Trabecular spacing (μm)	200 ± 5	136 ± 4	156 ± 4	148 ± 4.2[a]	0.0000

Data are mean±SE, n=5-9/group

[a] Leptin+ICI treated *ob/ob* mice different from vehicle treated WT mice, P≤0.05

[b] Leptin+ICI treated *ob/ob* mice different from vehicle treated *ob/ob* mice, P≤0.05

[c] Leptin+ICI treated *ob/ob* mice different from leptin treated *ob/ob* mice, P≤0.05

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Table 2

Effects of 1 month of daily sc leptin injection (40 μg/mouse/d) in the presence or absence of the potent antiestrogen ICI 182,780 (ICI) on cancellous bone architecture in lumbar vertebra in female *ob/ob* mice.

End Point	WT + Veh	*ob/ob* + Veh	*ob/ob* + Lep	*ob/ob* + Lep + ICI	ANOVA P Value
Bone volume/tissue volume (%)	14.5 ± 0.5	24.4 ± 1.2	20.9 ± 0.9	20.1 ± 1.4[a,b]	0.0000
Trabecular number (mm^{-1})	4.0 ± 0.1	5.6 ± 0.1	5.2 ± 0.2	5.4 ± 0.1[a]	0.0000
Trabecular thickness (μm)	43 ± 0	44 ± 1	44 ± 1	43 ± 1	0.4650
Trabecular spacing (μm)	255 ± 8	175 ± 5	189 ± 6	184 ± 3.4[a]	0.0000

Data are mean±SE, n=5-9/group

[a] Leptin+ICI treated *ob/ob* mice different from vehicle treated WT mice, P≤0.05

[b] Leptin+ICI treated *ob/ob* mice different from vehicle treated *ob/ob* mice, P≤0.05

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