Skeletal bone morphology is resistant to the high amplitude seasonal leptin cycle in the Siberian hamster

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Abstract

Recent studies have suggested that the adipocyte-derived hormone, leptin, plays a role in the regulation of metabolism. Here, we tested this hypothesis in the seasonally breeding Siberian hamster, as this species exhibits profound seasonal changes in adiposity and circulating leptin concentrations driven by the annual photoperiodic cycle. Male hamsters were kept in either long (LD) or short (SD) photoperiods. Following exposure to short photoperiods for 8 weeks animals exhibited a significant weight-loss and a 16-fold reduction of serum leptin concentrations. At Week 9, animals in both photoperiods were infused with leptin or PBS via osmotic mini-pump for 14 days. Chronic leptin infusion mimicked LD-like concentrations in SD-housed animals and caused a further decline in body

weight and adipose tissue. In LD-housed animals, leptin infusion resulted in a significant elevation of serum concentrations above natural LD-like levels, but had no discernable effect on body weight or overall adiposity. Both bending and compression characteristics and histomorphometric measurements of trabecular bone mass were unaltered by leptin treatment or photoperiod. Our data therefore show that despite a high natural amplitude cycle of leptin, this hormone has no apparent role in the regulation of bone metabolism, and therefore do not support recent propositions that this hormone is an important component in the metabolism of bone tissue.

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Introduction

The adipocyte-derived hormone, leptin, plays an important role in energy homeostasis (Schwartz *et al.* 2000). It has recently been suggested that in addition to its role in energy metabolism, this hormone also plays a significant role in the development and remodelling of bone. Here, the action of leptin may depend upon its route of administration. Ducy *et al.* (2000) have reported that obese mice deficient in leptin production (*ob/ob* genotype) or devoid of functional leptin receptor expression (*db/db* genotype) exhibit a markedly elevated bone mass with increased mineralization and bone formation rate compared with wild-type mice. Intracerebroventricular (i.c.v.) infusion of leptin in the *ob/ob* mice was shown to reverse this phenotype (Ducy *et al.* 2000). This has led to the recent view that in addition to its action on hypothalamic pathways controlling food intake, leptin may also act as a powerful inhibitor of bone formation.

Seasonally breeding mammals commonly exhibit substantial changes in the level of stored fat reserves and

circulating leptin concentrations (Rousseau *et al.* 2003). These adaptations are known to be driven by the seasonal daylength signal, which drives a hypothalamic relay involving the production of a daily pineal melatonin signal. This, in turn, acts on pathways that regulate the set-point for energy expenditure and food intake. As a result, seasonal mammals such as Siberian hamsters exhibit profound photoperiod-driven cycles of adiposity. Exposure to short winter daylength (SD) leads to a loss of up to 40% body weight, mostly in the form of fat (Steinlechner *et al.* 1983, Ebling 1994) and a 4–8 fold reduction in circulating leptin concentrations (Rousseau *et al.* 2003), compared with long photoperiod (LD).

We report experiments in which we tested the hypothesis that chronic exogenous leptin infusions would induce significant changes in bone metabolism. We infused leptin to both SD- and LD-housed animals at a dose designed to mimic natural levels observed on LD photoperiods, and then recorded the impact of these treatments on skeletal morphology.

Material and Methods

Animals

All animal procedures involving leptin infusions were licensed under the Animal (Scientific Procedures) Act of 1986, United Kingdom.

Adult male Siberian hamsters (*Phodopus sungorus*) from a colony bred at the University of Manchester and derived from animals described previously (Ebling 1994) were kept under controlled conditions (temperature, $21 \pm 1^\circ\text{C}$; humidity, 80%). All animals were individually housed within light-controlled environmental chambers lit by a 70-watt fluorescent white strip (100–400 lux) with continuous dim red light (<1 lux) throughout. Long-day (LD) photoperiods were 16 h light: 8 h darkness and short-day (SD) photoperiods were 8 h light: 16 h darkness. In the dark phase, a background illumination of dim red light was used (<1 lux).

Leptin infusion protocols

Recombinant murine leptin (supplied by Amgen Inc., Thousand Oaks, CA, USA) was dissolved in 0.01 M PBS and administered via a 7-day osmotic mini-pump (100 µl capacity; Alzet model 1007D, Charles River Laboratories UK, Kent, UK) to deliver a dose of 15 µg / day per animal over a 14-day period. Control animals received PBS vehicle alone. Pumps were implanted subcutaneously in the scapular region by sterile surgical procedure under halothane (Fluothane, AstraZeneca, Cheshire, UK) anaesthesia, as described previously (Atcha *et al.* 2000). The leptin infusions induced a similar serum concentration of leptin to that observed naturally under summer daylengths (circa 15 ng/ml) and causes significant fat and weight loss (Atcha *et al.* 2000).

Experimental protocols

Twenty-four weight-matched 16-week-old intact male hamsters reared in LD were individually housed and exposed to either LD or SD conditions for an 8-week period, as previously described (Atcha *et al.* 2000). At Week 9, each animal received an osmotic mini-pump containing either leptin or PBS (six animals per group per treatment). Pumps were replaced after 7 days for a further 1-week period. On Day 14 of treatment, a single blood sample was taken from each hamster under halothane anaesthesia by cardiac puncture for serum leptin determinations as previously described (Rousseau *et al.* 2002). Animals were then killed by cervical dislocation and the abdominal fat pads dissected and weighed. At the time of dissection, the carcasses were placed in 100% ethanol.

Measurement of trabecular bone mass

During dissection, vertebral tissue was maintained in ethanol and a strip of three thoracic vertebrae (T7 to T9)

removed. Tissue was dehydrated in ethanol, delipidised in chloroform and infiltrated in white glycol methacrylate resin (London Resin Co., Theale, Berkshire, UK), then polymerised in an anaerobic, temperature controlled environment. The tissue blocks were then orientated and sectioned coronally in the midpoint of the vertebral bodies. Five µm sections were stained with toluidine blue and mounted, using the standard methodologies employed in the Osteoarticular Pathology Laboratory, University of Manchester (Byers *et al.* 1999). Histomorphometric measurements were made on the T8 vertebral body using a Leica RMD microscope linked to a Leica QWin image analyser (Leica, Cambridge, UK). The vertebral body can be considered to be an oblong box attached to its neighbouring vertebrae at each end. The ends are actively growing and therefore possess growth plates. As growth plates are not examined in bone histomorphometry, our measurements excluded both the growth plate and the cortical bone, together known as the tissue area (TA). The amount of bone (trabecular bone area; TBA) was measured in each section and expressed as a percentage of TA ($(\text{TBA}/\text{TA}) \times 100$). Unless otherwise stated, all reagents supplied by WWR International Ltd., Poole, Dorset, UK.

Mechanical tests

Both bending and compression tests were performed using a universal mechanical testing machine (Instron model 4301, Instron, High Wycombe, UK).

Femora were subjected to three point bending tests. The femur was placed on two supports which were set 10 mm apart. A pushing probe of radius 20 mm was attached to the load cell and lowered until it just touched the mid-point of the sample. The crosshead was then lowered at a rate of 10 mm min^{-1} , bending the sample until it eventually broke. A computer with an interface to the testing machine was used to produce a graph of force vs displacement, permitting calculation of two mechanical properties of the femur: its bending strength and bending rigidity.

For the compression test, a strip of four lumbar vertebrae (L1 to L4) was removed in ethanol. Before the test, the vertebrae were individually dissected and rehydrated in saline overnight. Each lumbar vertebra was broken in compression along its longitudinal axis at a displacement rate of 5 mm/min. A load-displacement curve was recorded online and analysed for yield load (i.e. force causing the first damage of the vertebra).

Statistical analysis

Data were analyzed by *t*-tests, two-way ANOVA or two-way repeated measures of ANOVA followed by *post-hoc* Student–Newman–Keuls multiple comparison tests, using SigmaStat statistical software (SPSS, Chicago,

Figure 1 Effect of chronic leptin infusion on body weight (a), fat mass (b) and plasma leptin concentrations (c) in Siberian hamsters. a) shows the body weights measured at the beginning (Week 0; open bars) and at the end (Week 10; closed bars) of the experiment. Week 10 corresponds to the 8 weeks in either LD or SD photoperiods plus the 2 weeks of leptin infusion. Open bars represent the vehicle-treated (PBS) control groups and closed bars represent the leptin-infused groups (b and c). Values are the means $\pm$ S.E.M.

IL, USA). Results are presented as the means $\pm$ S.E.M. Differences were considered statistically significant at the $P < 0.05$ level.

Results

Effect of chronic leptin infusion on body weight, fat mass and serum leptin concentrations

Body weight and fat mass (Fig. 1a and b) were significantly

($P < 0.001$) reduced in SD (body weight (BW): 27.22 ± 0.57 g; fat mass: 0.54 ± 0.05 g) compared with LD (BW: 38.22 ± 1.48 g; fat mass: 2.62 ± 0.25 g) animals. In SD-housed animals, chronic leptin infusion mimicked LD levels (15.92 ± 4.3 ng/ml SD leptin versus 14.63 ± 6.01 ng/ml LD PBS; Fig. 1c), and caused further body weight (23.53 ± 1.06 g SD leptin versus 27.22 ± 0.57 g SD PBS; $P < 0.05$; Fig. 1a) and fat (0.12 ± 0.05 g SD leptin versus 0.54 ± 0.05 g SD PBS; $P < 0.001$; Fig. 1b) losses. Leptin infusion had no effect on LD-housed animals on either body weight (37.49 ± 1.02 g LD leptin versus 38.22 ± 1.48 g LD PBS; Fig. 1a) or fat mass (2.39 ± 0.16 g LD leptin versus 2.62 ± 0.25 g LD PBS; Fig. 1b).

Effect of chronic leptin infusion on bone mass and strength

Trabecular bone mass (TBA/TA %) was not significantly altered by photoperiod: $14.67 \pm 0.73\%$ in LD compared with $17.27 \pm 2.07\%$ in SD (Fig. 2a). Leptin infusion similarly did not affect trabecular bone mass in both LD ($17.20 \pm 2.55\%$ LD leptin versus $14.67 \pm 0.73\%$ LD PBS) and SD ($16.35 \pm 1.70\%$ SD leptin versus $17.27 \pm 2.07\%$ SD PBS) (Fig. 2a).

The mechanical properties of cortical bone were assessed by the three point bending test of the femur (Fig. 2b). Bending strength was not affected by photoperiod (31.96 ± 1.61 Newton metres $\times 10^{-3}$ in LD versus 32.28 ± 2.44 Newton metres $\times 10^{-3}$ in SD). Leptin infusion did not induce any significant change in bending strength in both LD (32.04 ± 2.57 Newton metres $\times 10^{-3}$ LD leptin versus 31.96 ± 1.61 Newton metres $\times 10^{-3}$ LD PBS) and SD (34.35 ± 2.74 Newton metres $\times 10^{-3}$ SD leptin versus 32.28 ± 2.44 Newton metres $\times 10^{-3}$ SD PBS). Similarly, neither leptin infusion nor photoperiod affected bending rigidity (data not shown).

The mechanical properties of trabecular bone were next investigated by a vertebral compression test (Fig. 2c). Vertebra compression strength was not affected by photoperiod (55.6 ± 3 Newtons in LD versus 52 ± 4.5 Newtons in SD). Leptin infusion did not induce any change in compression strength in both LD (50.10 ± 2.3 Newtons LD leptin versus 55.6 ± 3 Newtons LD PBS) and SD (55.3 ± 2.1 Newtons SD leptin versus 52 ± 4.5 Newtons SD PBS).

Discussion

Our data show that despite remarkable natural high amplitude changes in adiposity and circulating leptin concentrations, this was not associated with any significant changes in bone morphology. Our measurements examined both the morphometric and mechanical properties of bone, and neither were significantly altered by

Figure 2 Effect of chronic leptin infusion on bone mass and strength in Siberian hamsters. Trabecular bone mass of thoracic vertebra (a), femur bending strength (b) and compression strength of lumbar vertebra (c) were measured. Open bars represent the vehicle-treated (PBS) control groups and closed bars represent the leptin-infused groups. Values are the means $\pm$ S.E.M.

photoperiod change or chronic leptin infusions designed to mimic maximal long-day concentrations. As a consequence, we conclude that fluctuating leptin concentrations have no significant action on bone metabolism in this species.

Studies of laboratory rodents have recently provided some evidence for a role of leptin in bone re-modelling. Karsenty's laboratory reported that obese rodent models with defective leptin (*ob/ob*) or lacking a functional leptin receptor (*db/db*) displayed a markedly elevated bone mass (Ducy *et al.* 2000). When treated with recombinant leptin via a central (i.c.v.) route, both obese *ob/ob* mice and

normal mice exhibited significantly decreased bone mass (Ducy *et al.* 2000, Takeda *et al.* 2002, Elefteriou *et al.* 2004). Moreover, studies of two (*SAP-leptin* and *ApoE-leptin*) transgenic mouse strains showed that increasing serum leptin levels reduced bone mass (Elefteriou *et al.* 2004).

In our study, we have used a model species in which there are profound natural changes in leptin and adiposity driven by photoperiod, and have provided a 14-day signal designed to mimic the high levels achieved naturally on long photoperiods. We have shown elsewhere that long-day housed hamsters are refractory to these leptin infusion protocols and do not exhibit any significant feeding, reproductive or weight-loss response (Atcha *et al.* 2000). We have proposed that this resistance to leptin may be mediated at the level of the CNS (Rousseau *et al.* 2002). Here, our data clearly show that LD-housed animals do not respond to leptin in terms of any of the skeletal parameters measured. Similarly, SD-housed animals, despite differences induced by leptin in terms of metabolic responses, failed to show any significant effect of leptin infusion on bone mass and strength. Thus, our studies clearly indicate that both naturally induced changes and exogenous chronic infusions of this hormone are ineffective in causing skeletal re-modelling. There were no obvious differences in the morphology of vertebral bone in our hamster material compared with laboratory rodents such as mice or rats.

Several laboratories have published contradictory evidence for a role of leptin in bone metabolism. In contrast to studies on mice, obese *fa/fa* rats, which have a mutated leptin receptor, are reported to have a decreased bone mass (Foldes *et al.* 1992, Picherit *et al.* 2003). Models in which leptin has been administered centrally via an i.c.v. route have shown a reduced bone mass (Ducy *et al.* 2000, Takeda *et al.* 2002, Elefteriou *et al.* 2004). However, models employing peripheral administration of leptin have been reported to increase bone growth in *ob/ob* mice (Liu *et al.* 1997, Steppan *et al.* 2000) and to reduce the extent of bone loss in ovariectomized rats (Burguera *et al.* 2001), while systemic administration of leptin to adult male mice has been reported to increase bone strength (Cornish *et al.* 2002). It has been proposed that the paradoxical differences between central and peripheral effects of leptin on bone re-modelling in laboratory rodents are due to the effects of high peripheral levels leading to a state of central leptin resistance, due perhaps to saturation of receptors (Khosla 2002).

In humans, some *in vitro* studies clearly demonstrate a local and direct effect of leptin on human osteoblasts. Indeed, expression of leptin and its receptor was shown in primary cultures of normal human osteoblasts (Reseland *et al.* 2001), while exogenous leptin promoted osteoblastic cell proliferation and differentiation as well as bone mineralization (Reseland *et al.* 2001, Gordeladze *et al.* 2002). Nevertheless, other data are inconclusive. In

cross-sectional studies, although some authors reported a positive association between serum leptin levels and bone mineral density, others failed to find such a relationship or even observed a negative relationship (for a review see Thomas 2004). Those few interventional studies conducted in humans have also produced conflicting outcomes. For instance, subcutaneous leptin therapy for up to four years in three morbidly obese children congenitally deficient in leptin did restore bone mass to normal age-related development (Farooqi *et al.* 2002). However, two groups (Simha *et al.* 2002, Moran *et al.* 2004) have reported that in patients with generalized lipodystrophy, chronic exogenous leptin administration had no impact on bone mass density.

In conclusion, our studies do not support recent investigations in rodents that have reported the action of leptin on bone metabolism. Using a leptin infusion model, our data suggest that in wild seasonally breeding animals adaptations may exist to ensure that skeletal characteristics are able to resist seasonal changes in energy metabolism and circulating leptin concentrations. Our data therefore cast some doubt on the proposition that leptin may play an important general role in the metabolism of bone in species other than laboratory rats and mice.

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