

Leptin directly regulates bone cell function *in vitro* and reduces bone fragility *in vivo*

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Abstract

Fat mass is an important determinant of bone density, but the mechanism of this relationship is uncertain. Leptin, as a circulating peptide of adipocyte origin, is a potential contributor to this relationship. Recently it was shown that intracerebroventricular administration of leptin is associated with bone loss, suggesting that obesity should be associated with low bone mass, the opposite of what is actually found. Since leptin originates in the periphery, an examination of its direct effects on bone is necessary to address this major discrepancy.

Leptin ($>10^{-11}$ M) increased proliferation of isolated fetal rat osteoblasts comparably with IGF-I, and these cells expressed the signalling form of the leptin receptor. In mouse bone marrow cultures, leptin ($\geq 10^{-11}$ M) inhibited osteoclastogenesis, but it had no effect on bone resorption in two assays of mature osteoclasts. Systemic administration of leptin to adult male mice (20 injections

of 43 $\mu\text{g}/\text{day}$ over 4 weeks) reduced bone fragility (increased work to fracture by 27% and displacement to fracture by 21%, $P<0.001$). Changes in tibial histomorphometry were not statistically significant apart from an increase in growth plate thickness in animals receiving leptin. Leptin stimulated proliferation of isolated chondrocytes, and these cells also expressed the signalling form of the leptin receptor.

It is concluded that the direct bone effects of leptin tend to reduce bone fragility and could contribute to the high bone mass and low fracture rates of obesity. When administered systemically, the direct actions of leptin outweigh its centrally mediated effects on bone, the latter possibly being mediated by leptin's regulation of insulin sensitivity.

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Introduction

Low body weight has long been recognised as one of the common clinical associations of osteoporosis (Felson *et al.* 1993). Epidemiological studies have demonstrated that low body weight is a risk factor for fractures of both the proximal femur and the vertebrae (Gardsell *et al.* 1989, Hassager & Christiansen 1989, Greenspan *et al.* 1994). The advent of bone densitometry permitted the demonstration that bone density itself is also closely related to body weight (Mazess *et al.* 1987). Dual-energy X-ray absorptiometry permits body weight to be divided into its fat and lean components. Using this technique, it has become clear that fat mass is a major correlate of bone density (Reid *et al.* 1992a,b), of the rate of postmenopausal bone loss (Reid *et al.* 1994a), and of fracture incidence (Schott *et al.* 1998).

The biological basis for the relationship between fat mass and bone density remains uncertain. It has been

attributed to the biomechanical effect of load bearing on the skeleton. While this may contribute, it is not a complete explanation, since the correlations between fat mass and bone density in non-weight-bearing parts of the skeleton are comparable with those found axially (Reid *et al.* 1992a). Furthermore, in some studies the impact of fat mass on bone density has been found to be greater than that of lean mass, whereas if it was simply a load-bearing phenomenon the two would be expected to contribute equally (Compston *et al.* 1992, Reid *et al.* 1992a, 1994b, Khosla *et al.* 1996). A second explanation is that adipocytes are a major site of oestrogen production in postmenopausal women. Again, this is unlikely to be the whole explanation (van Beresteijn *et al.* 1992) and it does not explain the close relationship between fat mass and bone density in premenopausal women despite the insignificance of the adipocyte as a source of oestrogen in this age group (Reid *et al.* 1992b). A third possibility is that obesity influences bone density by way of its effects on circulating

concentrations of bone-active hormones. Fat mass is a major determinant of circulating insulin concentrations and there is clear evidence that insulin acts both directly and indirectly on the skeleton (Reid *et al.* 1993, Cornish *et al.* 1996). Amylin, a hormone co-secreted with insulin from the beta cells of the pancreatic islets, also directly stimulates osteoblast activity and inhibits osteoclastic bone resorption (Cornish & Reid 1999). There may be other hormones co-secreted from the beta cell or directly from the adipocyte that are also involved.

The identification of leptin as a major hormonal product of the adipocyte has raised the possibility that this peptide may contribute to the fat mass–bone density relationship. This would be possible if this hormone acted on bone. To date, it has been demonstrated that circulating leptin concentrations are related to bone mass (Goulding & Taylor 1998, Pasco *et al.* 2001, Yamauchi *et al.* 2001, Blain *et al.* 2002), and that leptin levels are lower in women with vertebral fractures (Yamauchi *et al.* 2001), although association does not establish causality. Leptin has been shown to promote osteoblastic differentiation (Thomas *et al.* 1999) in a human osteoblast cell line but this has been contradicted by the recent work of Ducy *et al.* (2000), who concluded that there was no direct action of leptin on skeletal cells. In contrast, they demonstrated profound bone loss when leptin was administered into the cerebral ventricles of either wild-type or leptin-deficient mice. These experiments demonstrated that bone density was centrally regulated but left a major unresolved paradox, since they suggested that high leptin states such as obesity would be associated with low bone density, whereas the opposite is the case.

While the hypothalamus is an important site of leptin action, leptin originates outside the central nervous system, in the adipocyte. Therefore, to determine its effects on the skeleton of the intact animal, it is necessary to study the effects of its systemic administration – if it does have direct effects on bone, these will modify those arising from its central actions. The present paper addresses these questions by assessing the effects of leptin on skeletal cells *in vitro*, as well as assessing its integrated action following systemic administration *in vivo*.

Materials and Methods

Osteoblast-like cell culture

Osteoblasts were isolated from 20-day-old fetal rat calvariae as previously described (Cornish *et al.* 1999). Briefly, calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. Usually, two litters were used for each osteoblast preparation. The calvariae were sequentially digested using collagenase and the cells from digests three and four were collected, pooled and washed. Cells were grown to

confluence and then subcultured into 24-well plates. This preparation produces cells with a pre-osteoblast phenotype, evidenced by alkaline phosphatase expression but low basal expression of osteocalcin (Groot *et al.* 1985, Herrmann-Erlee & van der Meer 1986). Cells were growth arrested in minimum essential medium (MEM) (Gibco BRL, Life Technologies, Auckland, NZ)/0.1% BSA for 24 h. Fresh medium and experimental compounds were added for a further 24 h. Cells were pulsed with [³H]thymidine 2 h before the end of the experimental incubation. The experiment was terminated and both cell counts and thymidine incorporation were assessed. There were six wells in each group and each experiment was repeated three or four times. For the long-term cultures (10 and 21 days) the cells were grown initially as above. At the point of confluency, medium was supplemented with 50 mg/ml ascorbic-2-phosphate and 10 mM β-glycerol phosphate to promote differentiation.

Identification of leptin receptors

Leptin receptors were identified by RT-PCR using osteoblast-like cells, prepared as above. Total cellular RNA was purified from cells and tissues by a modified method of single-step guanidinium thiocyanate–phenol–chloroform RNA extraction (Chomczynski & Sacchi 1987, Grey *et al.* 2001). RNA concentration and purity were determined by measuring the optical density using a Gene Quant™ spectrophotometer (Pharmacia, Little Chalfont, Bucks, UK) and the quality was determined by electrophoresis on a 1% agarose gel. RNA was treated with DNase and RT-PCR amplifications were carried out following the previously published protocol (Grey *et al.* 2001). PCR was performed in an automatic DNA thermal cycler (Mastercycler Personal; Eppendorf, Hamburg, Germany). After an initial denaturation step of 2 min at 94 °C, cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min were performed. PCR reaction products were visualised on a 1% TBE agarose gel. The primers used to amplify the leptin receptor (long form) from rat and human RNA were 5'GGTCCTCTTCTTTGGAGCCTG and 5'GCTGGGAATGGGCACGATAC. For amplification of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the forward primer 5'CATCATCTCCGCCCTCCG and the reverse primer 5'CCTGCTTACCACCTTCTTG were used, while for the human GAPDH the forward primer 5'CATCATCTCTGCCCTCTG was used with the same reverse primer as for the rat GAPDH. Twenty-five amplification cycles were performed for the GAPDH, and 35 cycles for the leptin receptor. PCR products were purified from agarose gels using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), and their sequences were determined on an ABI 377 XL DNA Sequencer (PE Biosystems, Foster City, CA, USA).

Bone marrow culture

Bone marrow was obtained from long bones of normal Swiss male mice aged 4–6 weeks. Approximately six animals were used per preparation. Mice were killed by cervical dislocation while under halothane anaesthesia. Femurs and tibiae were aseptically removed and dissected free of adhering tissues. The epiphyses were cut off with a scalpel blade and the marrow cavity was flushed with alpha minimum essential medium (α MEM) using a syringe with a 23-gauge needle. The marrow cells were collected in a 50 ml centrifuge tube, centrifuged at 232 *g* for 2 min and washed with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA)/ α MEM. Marrow cells were then cultured for 2 h in 90 mm Petri dishes. After 2 h, non-adherent cells were collected, centrifuged at 232 *g* for 2 min, washed with 15% FBS/ α MEM and seeded at 10^6 cells/ml in 48-well plates (Costar, Cambridge, MA, USA) (0.5 ml/well). 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D) (10^{-8} M) was added (day 0) to all wells except to negative controls, which remained in the absence of 1,25(OH)₂D throughout the experiment. Forty-eight hours later (day 2), cultures were fed 0.5 ml fresh medium with 1,25(OH)₂D to make a total of 1 ml/well. After a further 48 h (day 4), cultures were fed by replacing 0.5 ml old medium with fresh medium with 1,25(OH)₂D. Test substances were added to test groups and vehicle added to control groups at 0, 2, 4 days, depending on the particular protocol. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. After culture for 7 days, cells adherent to the well surface were fixed with citrate–acetone–formaldehyde (25:65:8 v/v) for 30 s. Culture plates were then stained for tartrate-resistant acid phosphatase (TRAP) using Sigma Kit #387-A (Sigma Diagnostics, St Louis, MO, USA). TRAP-positive multinucleated cells (containing three or more nuclei) were counted in all wells. Each experiment had three wells in which cells were grown on bone slices and checked for resorptive pits, indicating that the TRAP-positive multinucleated cells in these cultures were capable of resorbing bone. There were at least eight wells for each group and each experiment was repeated three or four times.

Mature isolated osteoclast culture

Rat osteoclasts were isolated from a litter of 1-day-old neonatal rats. The rats were killed by decapitation, and the long bones aseptically removed. The bones were dissected free of adherent soft tissues. Epiphyses were removed and the remaining diaphyses split longitudinally and then placed in a tissue homogeniser containing 2 ml acidified α MEM (72 μ l concentrated HCl per 80 ml medium) with 10% FBS and antibiotics. Bones were homogenised gently and the cell suspension collected in a 15 ml conical tube. The remaining bone tissue was placed in a small Petri dish with 1 ml medium and chopped quickly with a scalpel

blade. The resulting cell suspension was collected and added to the same conical tube. The bone tissue was homogenised again in 1 ml medium and the suspension was once again collected. This osteoclast-rich suspension was placed onto bovine bone slices, approximately 9 mm², in 96-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for 35 min to allow the mature osteoclasts to settle. The bone slices were washed several times in PBS to remove contaminating non-osteoclastic cells followed by a rinse in medium. The bone slices were then placed in six-well plates (four slices per well) containing 6 ml medium and incubated with test substances or vehicle for 20 h. The identity of cells produced in this way as mature osteoclasts is attested to by their ability to resorb bone, by the presence of calcitonin receptors, by positive TRAP staining and by their being multinucleated.

After incubation the bone slices were fixed with 2.5% glutaraldehyde/PBS and stained for TRAP using Sigma kit #387-A. The number of TRAP-positive multinucleated (containing more than three nuclei) cells on each bone slice was quantified and the cells removed by gentle scrubbing and then stained for 30 s with toluidine blue. After numerous washes in water the bone slices were dried and then assessed for the 'pits' excavated by the osteoclasts. This was achieved using reflected light microscopy and metallurgic lenses. The results were expressed as a ratio of the number of pits: number of osteoclasts per bone slice. There were 6–12 bone slices in each group and each experiment was repeated two or three times.

Bone organ culture

Bone resorption studies were carried out in neonatal mouse calvariae as described previously (Reid *et al.* 1990). Mice were injected s.c. with 5 μ Ci ⁴⁵Ca at 2 days of age, and hemi-calvariae were dissected out 4 days later. Hemi-calvariae were pre-incubated for 24 h in medium 199 with 0.1% BSA, then changed to fresh medium containing test substances or vehicle. Incubation was continued for a further 48 h. To assess DNA synthesis, [³H]thymidine (0.6 μ Ci/ml) was added in the last 4 h of the incubation as described previously (Lowe *et al.* 1991). The experiment was terminated and both calcium release and thymidine incorporation were assessed. There were five to seven hemi-calvariae in each group and each experiment was repeated three or four times.

Chondrocyte cell culture

Chondrocytes were isolated by removing cartilage (full-depth slices) from the tibial and femoral surfaces of adult dogs under aseptic conditions. Slices were placed in Dulbecco's Modified Eagle's medium (DMEM) containing 5% FBS (v/v) and antibiotics (penicillin 50 U/l,

streptomycin 50 µg/l and neomycin 100 µg/l) and chopped finely with a scalpel blade. Tissue was removed and incubated at 37 °C with pronase (0.8% w/v for 90 min) followed by collagenase (0.1% w/v for 18 h) to complete the digestion. The cells were isolated from the digest by centrifugation (10 min at 272 g), resuspended in DMEM/5%FBS, passed through a nylon mesh screen of 90 µm pore size to remove any undigested fragments, and recentrifuged. The cells were washed and resuspended twice in the same medium and seeded into a 75 cm² flask containing DMEM/10% FBS/50 µg/ml ascorbic acid. The cells were incubated under 5% CO₂/95% air at 37 °C. Confluence was reached by 7 days, at which time the cells were subcultured. After trypsinisation using trypsin–EDTA (0.05%/0.53 mM), the cells were rinsed in DMEM/5% FBS/50 µg/ml ascorbic acid and resuspended in fresh medium, then seeded into 24-well plates (5 × 10⁴ cells/ml, 0.5 ml/well). Measurement of cell numbers and thymidine incorporation were performed in growth-arrested cell populations as for the osteoblast-like cell cultures. For the human chondrocyte cultures, articular cartilage was obtained from knee joints of 50- to 70-year-old patients undergoing joint replacement. The cartilage slices excised were from non-diseased areas and tissue appeared normal. The chondrocytes were isolated by pronase/collagenase digestion as above.

Systemic study

Experimental design Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25–36 g, were given daily s.c. injections (43 µg recombinant murine leptin in 50 µl water, or water alone) in the loose skin at the nape of the neck for 5 days/week over 4 consecutive weeks. This dose was chosen because the same molar doses (93 nmol/kg) of amylin and adrenomedullin in this model produce substantial effects on bone turnover and bone area (Cornish *et al.* 1998, 2001). These peptides have similar effects on osteoblast proliferation to leptin (*vide infra*). Animals were housed in a room maintained at 20 °C on 12 h light:12 h darkness cycles. They were fed diet 86 rodent pellets (NRM NZ Ltd, Takanini, New Zealand) freely throughout the experiment. Each animal's weight was recorded at the beginning and end of the experiment. The study had the approval of the local institutional review board.

Histomorphometry Indices of bone formation and resorption, and bone volume were assessed in the proximal tibiae. One tibia from each animal was used for histomorphometric analyses. The tibiae were dissected free of adherent tissue and bone lengths were recorded by measuring the distance between the proximal and the distal epiphysis using an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Tibiae were then processed as previously described (Cornish *et al.* 2000). Briefly, bones

were fixed in 10% phosphate-buffered formalin for 24 h and then dehydrated in a graded series of ethanol solutions and embedded, undecalcified, in methylmethacrylate resin. Tibiae were sectioned longitudinally through the frontal plane. Sections (4 µm thick) were cut using a rotary microtome and a tungsten carbide knife, then mounted on gelatin-coated slides and air-dried. They were stained with Goldner's tri-chrome and examined using an Olympus BX 50 microscope (Olympus, Tokyo, Japan), which was attached to an Osteomeasure Image Analyzer (Osteometric, Atlanta, GA, USA).

Tibial histomorphometric analyses were made from three adjacent sections one-third of the way through the anterior/posterior depth of the proximal tibiae. All trabecular bone tissue in the secondary spongiosa was quantified for bone volume in each section using a × 10 objective, and parameters were derived using the formulae of Parfitt *et al.* (1983). Parameters of bone formation and resorption were measured using a × 20 objective in all trabecular bone tissue in the secondary spongiosa in the second of the three adjacent sections. The surfaces measured were those immediately adjacent to unmineralised matrix, those adjacent to osteoblasts, and those adjacent to osteoclasts (osteoid, osteoblast and osteoclast perimeters respectively). Perimeters and cell numbers were expressed per section. Cortical width was measured on both sides of the tibial shaft, 2.5 mm below the epiphyseal growth plate. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator (J C) who was blinded to the treatment group of each bone. The precisions of these histomorphometric measurements in our laboratory (expressed as coefficients of variation of paired measurements) are as follows: mineralised bone area 1.3%, osteoid perimeter 6.9%, osteoblast perimeter 6.8%, osteoblast number 1.7%, osteoclast perimeter 7.9%, osteoclast number <1.0%, width measurements 1.7%.

Mechanical assessment of the tibia The remaining tibia from each animal, stored frozen, was thawed and its mechanical properties assessed by means of a three-point bending test. Each tibia was dissected free of soft tissue and tested on a universal testing machine (Model 1186; Instron, Canton, MA, USA) equipped with a 5000 N load transducer. Samples were tested at room temperature with a support span of 10 mm. Appropriately shaped beam end supports were used to minimise twisting of the bone about its long axis. Load was applied at a constant deformation rate of 2 mm/min. Load–deformation curves were recorded and displacement values (a measurement of how much the bone deflects in bending up to point of fracture) were obtained directly from the curve and expressed in millimetres.

Fat mass estimations Fat mass estimations were made from measurements of the animals' body densities

calculated from water displacement. Immediately after death the mice were submerged head-first to the base of the tail into a 250 ml measuring cylinder containing 150 ml water and the displacement volume recorded. The fraction of body weight that was fat mass was calculated using a modification of the Siri equation for use in rodents (Muscaritoli *et al.* 1993). The coefficient of variation for repeated measures of fat mass was 7%.

Statistical analysis

Data are presented as means \pm S.E. Where parameters were measured more than once in each animal (e.g. cortical thickness), these values were averaged to produce a single value for each animal before further analysis. The significance of treatment effects was evaluated using Student's *t*-tests for unpaired data and a 5% significance level was maintained.

Results

Osteoblasts

Leptin produced a dose-related increase in both thymidine incorporation and cell number in primary cultures of osteoblast-like cells at 24 h (Fig. 1a, 1b). Both effects were statistically significant at leptin concentrations of 10^{-10} M, although cell number were already showing an upward trend at 10^{-11} M. For purposes of comparison, the effects of maximally effective concentrations of a number of classic osteoblast mitogens are also shown in Fig. 1c. It can be seen that the effects of leptin are of comparable magnitude. Leptin mRNA was not detected using RT-PCR in these osteoblast cultures (data not shown).

Leptin receptors

In view of the activity of leptin on isolated osteoblasts, we determined whether these cells expressed the signalling form of the leptin receptor. Figure 2a shows results from a representative study using semi-quantitative RT-PCR. mRNA for the leptin receptor was detectable in these cells, although its level of expression was lower than in rat hypothalamic RNA that was used as a positive control. The expression of the leptin receptor was also studied in isolated osteoblasts that were cultured for 5 or 10 days, and in cells cultured for 21 days, which are more differentiated and are capable of forming bone nodules. As shown in Fig. 2b, the levels of expression of the leptin receptor were similar in all these RNA samples.

Osteoclasts

The effects of leptin on osteoclast development were assessed in mouse bone marrow cultures. The number

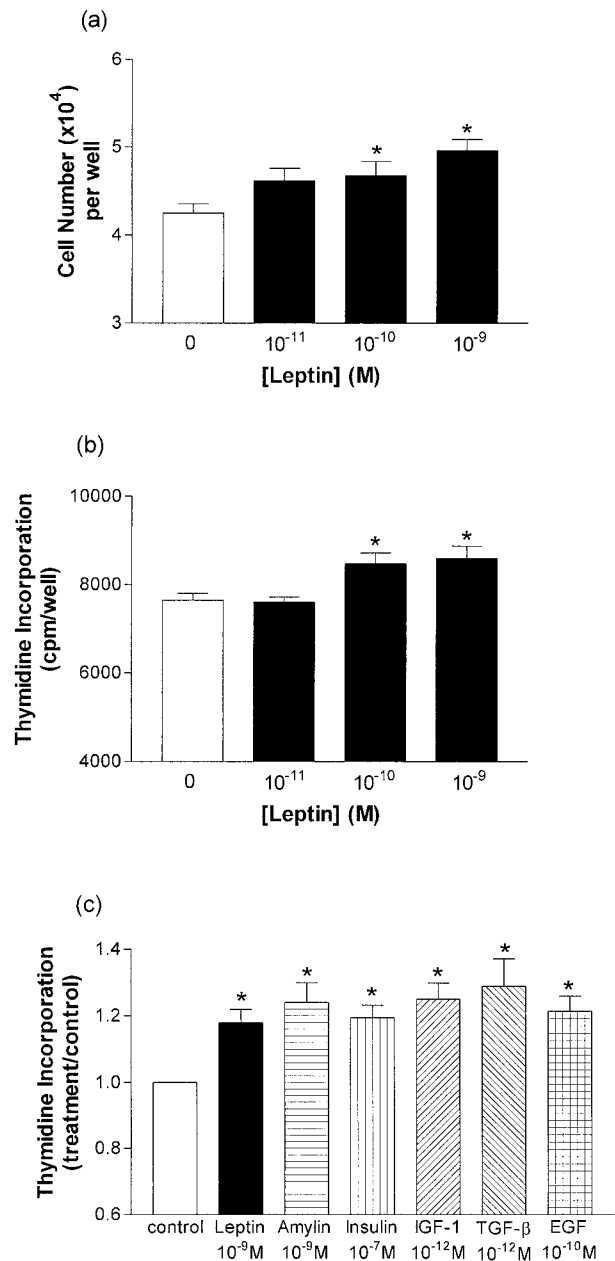


Figure 1 Effect of leptin on: (a) cell number, and (b) thymidine incorporation in primary cultures of fetal rat osteoblasts at 24 h. In (c), the response to a maximal dose of leptin is compared with those to maximal concentrations of a number of other osteoblast growth factors (IGF-1, insulin-like growth factor; TGF-β, transforming growth factor-β; EGF, epidermal growth factor). Means \pm S.E.M. Data pooled from more than one experiment are shown as treatment/control ratios. *Significantly different from control; $P \leq 0.05$ in (a), $P \leq 0.01$ in (b, c).

of multinucleated, TRAP-positive cells was significantly decreased by leptin concentrations as low as 10^{-11} M (Fig. 3a). These effects are comparable in magnitude with

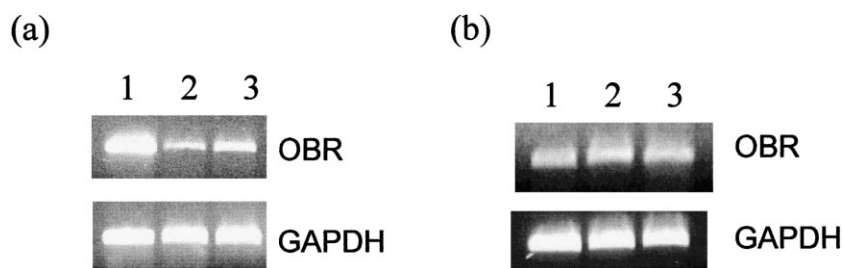


Figure 2 (a) Expression of the long (signalling) form of the leptin receptor assessed by RT-PCR. Lane 1, rat hypothalamus; lane 2, rat primary osteoblast culture; lane 3, human chondrocyte culture. (b) Expression of the leptin receptor in rat primary osteoblasts after 5 days (lane 1), 10 days (lane 2) and 21 days (lane 3) in culture. OBR, long form of leptin receptor.

those we have shown with weak anti-osteoclast factors such as amylin, but very much less than those observed with calcitonin (data not shown).

In contrast, leptin had no effect in two models of mature osteoclast activity. Pit formation on bovine bone by isolated mature rat osteoclasts was unaffected by leptin in concentrations as high as 10^{-8} M (Fig. 3b). Similarly, calcium release from cultured neonatal mouse calvariae was unaffected by multiple additions of leptin in concentrations as high as 10^{-7} M. Findings were similar whether the effect of leptin was assessed on basal resorption or on resorption stimulated by parathyroid hormone (Fig. 3c).

In vivo

Normal adult male mice were treated systemically with leptin or vehicle over a 4 week period. Histomorphometry data from the proximal tibiae of these animals are given in Table 1 – the only significant difference between the groups was in growth plate thickness, which was greater in the animals treated with leptin. However, when bone mechanical properties were assessed by three-point bending, both the work to failure and the displacement to failure were significantly increased in the animals treated with leptin (Fig. 4).

Weight increased from 27.5 ± 0.8 to 33.6 ± 0.8 g in the course of the study in animals treated with vehicle,

Table 1 Effects of leptin treatment on bone histomorphometry. Data are means \pm S.E.M.

Parameter	Control	Leptin
Osteoblast number	600 \pm 50	640 \pm 40
Osteoid perimeter (mm)	5.0 \pm 0.6	6.2 \pm 0.7
Osteoclast number	8.0 \pm 0.8	7.3 \pm 0.7
Bone volume (%)	19.0 \pm 1.3	18.0 \pm 1.4
Cortical thickness (mm)	0.189 \pm 0.005	0.197 \pm 0.008
Growth plate thickness (mm)	0.083 \pm 0.004	0.099 \pm 0.004*
Mid-tibial width (mm)	0.30 \pm 0.02	0.32 \pm 0.02

*Significantly different from control, $P=0.01$.

and those receiving leptin showed comparable changes (27.2 ± 1.0 to 32.5 ± 1.0 g). The per cent fat mass of the animals at the end of the experiment was lower in the leptin animals ($8.7 \pm 0.3\%$) than in the control group ($10.2 \pm 0.5\%$, $P=0.01$). Tibial lengths were not different between the groups (control 18.6 ± 0.1 mm, leptin 18.7 ± 0.0 mm).

Chondrocytes

In view of the effects of leptin administration on growth plate thickness, its effect on proliferation of primary cultures of canine chondrocytes was also assessed. Leptin at 10^{-10} M increased numbers of chondrocytes from $3.11 \pm 0.08 \times 10^4$ in control cultures to $3.36 \pm 0.06 \times 10^4$ ($P<0.05$). Thymidine incorporation showed a similar response: control, 23 704 \pm 288 d.p.m./well; leptin, 25 708 \pm 396 d.p.m./well ($P=0.002$). Similar results were obtained from a preparation of ovine chondrocytes (data not shown). The expression of the leptin receptor was demonstrated in RNA from a primary human chondrocyte culture using RT-PCR (Fig. 2a, lane 3).

Discussion

The present studies assess the skeletal actions of leptin in a number of different models, and indicate that it has modest direct effects on bone cells leading to reduced bone fragility. Leptin promotes growth in cultures of both primary osteoblasts and chondrocytes. This anabolic effect is consistent with the data from Thomas *et al.* (1999) in a human marrow stromal cell line indicating that leptin promoted differentiation into an osteoblast phenotype and that it increased synthesis of bone matrix proteins such as type I collagen and osteocalcin. Similar findings have been reported by Gordeladze *et al.* (2002). The present findings are also consistent with the data from Steppan *et al.* (2000) who found that the *in vivo* administration of leptin to ob/ob mice resulted in increases in both bone size and

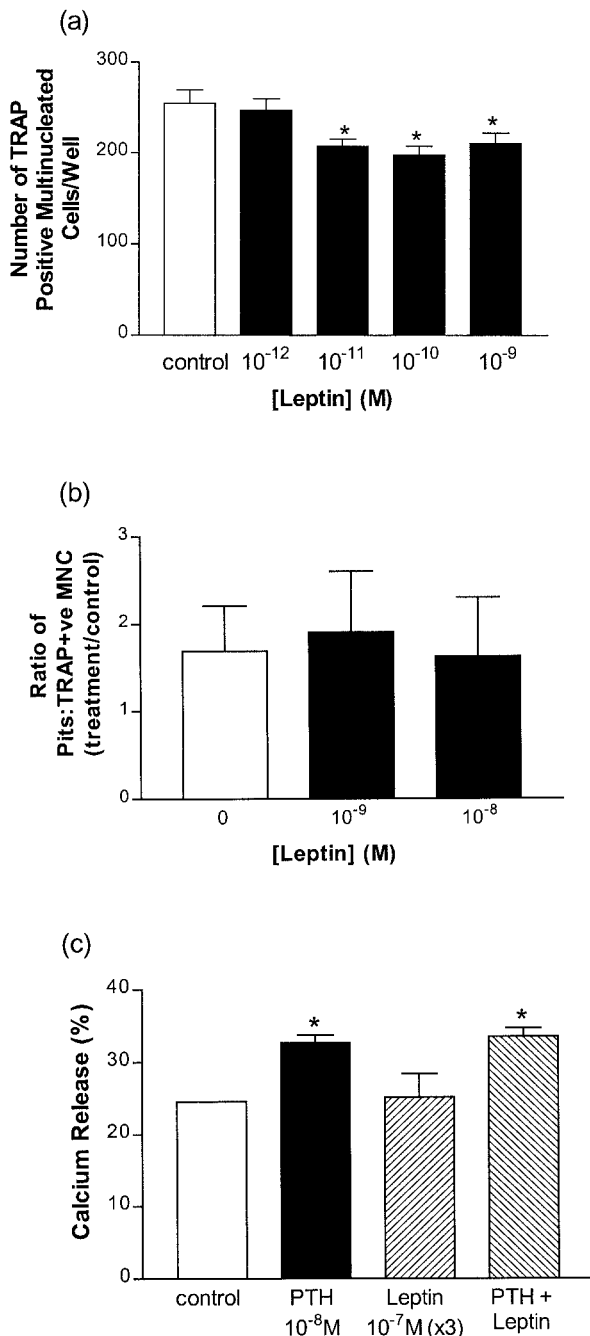


Figure 3 (a) Effect of leptin on osteoclast development in mouse bone marrow cultures. The number of multinucleated, TRAP-positive cells was significantly decreased by leptin. *Significantly different from control, $P < 0.04$. (b) Effect of leptin on pit formation on bovine bone by isolated mature rat osteoclasts. No significant effects were detected. (c) Effect of leptin on basal calcium release and that stimulated by 10^{-8} M parathyroid hormone (PTH) in cultured neonatal mouse calvariae. Leptin was added in a concentration of 10^{-7} M three times during the 48 h incubation period. There were no significant effects of leptin on calcium release. *Significantly different from control, $P < 0.008$. Means \pm S.E.M.

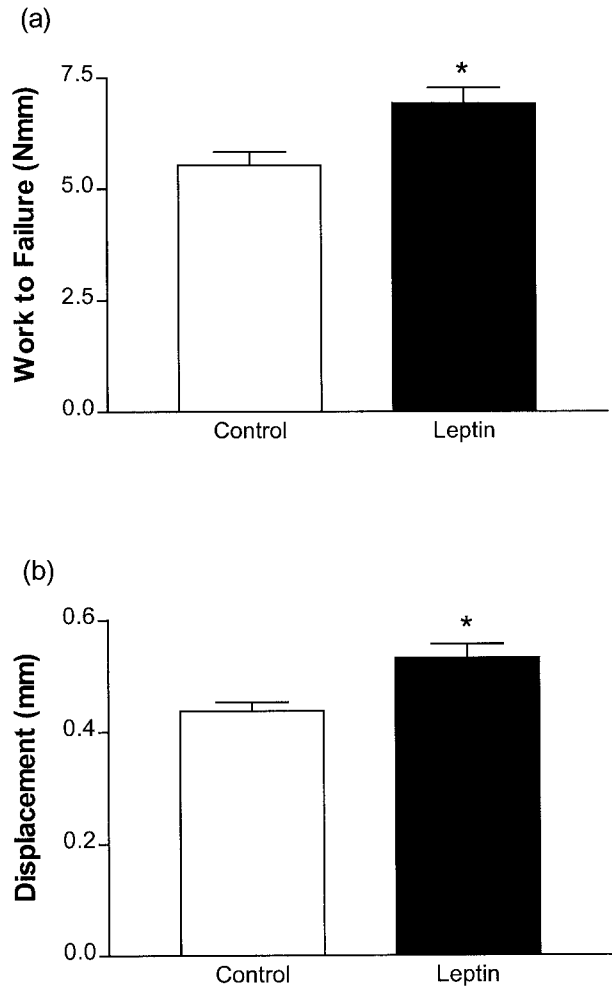


Figure 4 Effect of leptin on bone fragility in the tibia of normal adult mice treated with leptin at 43 μ g/day for 4 weeks, or vehicle. Means \pm S.E.M. The indices shown were determined from load–deformation curves during three-point bending. *Significantly different from control, $P < 0.001$.

bone mass. A similar *in vivo* study in *ob/ob* mice, published in abstract form only, indicates that leptin replacement increases osteoblast number more than 20-fold, with accompanying substantial increases in bone formation rates (Liu *et al.* 1997). Also in agreement with the present results, Iwaniec *et al.* (1998) have provided a preliminary report of dose-related increases in the number of mineralised bone nodules formed *in vitro* after 14 days of treatment of primary osteoblast cultures with leptin in concentrations between 1 and 100 ng/ml. These results have been confirmed by Reseland *et al.* (2001). Together these studies provide a compelling body of evidence that leptin has direct anabolic effects on osteoblasts. This is supported by our demonstration of the signalling form of the leptin receptor in primary rat osteoblast cultures and human chondrocytes. This receptor has also been found in

human osteoblasts (Steppan *et al.* 2000, Enjuanes *et al.* 2002), rat osteoblasts (Lee *et al.* 2001), porcine chondrocytes (Steppan *et al.* 2000), human mesenchymal stem cells undergoing osteogenic differentiation (Bassilana *et al.* 2000), and in osteoblastic and chondrocytic cell lines (Sufang *et al.* 2000, Lee *et al.* 2001), supporting the present evidence that leptin plays a role in skeletal physiology.

Leptin also acts on bone resorption, reducing osteoclast formation in mouse bone marrow cultures. This is consistent with the finding of an inverse relationship between serum leptin and bone resorption markers in human fetuses (Oguchi *et al.* 2000) and in postmenopausal women (Blain *et al.* 2002), and with the report from Holloway *et al.* (2002) of an inhibition of osteoclast generation in an assay using human peripheral blood mononuclear cells. Those studies suggested that the effect was mediated by leptin-induced changes in mononuclear cell production of receptor activator of NF- κ B (RANK), and are complemented by the report of Burguera *et al.* (2001) that leptin increases osteoprotegerin levels and decreases RANK ligand levels in human marrow stromal cells. Indeed, leptin may have a wider role in the regulation of bone marrow cells, since there is also evidence that it stimulates haematopoiesis and immune cell generation (Pighetti *et al.* 1999). However, the present studies indicate that leptin does not act on mature osteoclasts, whether assessed in isolated cells or in an organ culture system in which there is very little osteoclast generation.

This is the first study to assess the effects of systemic leptin administration on bone histomorphometry and strength in wild-type animals. The upward trends in osteoblast indices and the downward trend in osteoclast number reflect the changes seen in the *in vitro* studies. They may be smaller *in vivo* because of osteoblast-osteoclast coupling and the fact that leptin exerts opposite effects on formation and resorption. Burguera *et al.* (2001) also found no effects of leptin on forming and resorbing surfaces in ovariectomised rats, although they did find a reduction in trabecular bone loss. The significant reduction in skeletal fragility with leptin in our own studies is consistent with the *in vitro* effects of this hormone and is the more impressive since it occurs in the face of reduced body weight caused by leptin's central effect on appetite (Halaas *et al.* 1995). It is surprising that significant changes are not seen in the histomorphometric indices. This may reflect the relative insensitivity of histological measures, but it could also suggest that the effect on fragility is mediated by changes in bone architecture. In particular, strength in three-point bending is related to the fourth power of the external diameter of a bone, so the small increases in cortical thickness and mid-tibial width in the leptin-treated animals are sufficient to account for the observed changes in the bones' mechanical properties.

The positive effects of leptin on bone size, growth plate thickness and chondrocyte proliferation in the present studies suggest that it regulates bone growth. This is

consistent with the reduced bone size of leptin-deficient mice (Steppan *et al.* 2000) and leptin-resistant rats (Mathey *et al.* 2002), and with evidence of a relationship between bone size and circulating leptin concentrations in adolescent women (Matkovic *et al.* 1997). It is also consistent with the recent report of Maor *et al.* (2002) that leptin directly promotes chondrocyte growth. Two studies of systemic leptin administration in ob/ob mice show dramatic positive effects on indices of bone formation (Liu *et al.* 1997), bone size and bone density (Steppan *et al.* 2000). These studies showed larger effects than the present study, probably because they were carried out in leptin-deficient mice, although the younger age of the animals and the higher leptin doses used in those experiments may also have contributed. The positive effect of leptin on skeletal size suggests that this hormone may be a contributor to the increases in stature of successive generations of humans that have been observed over the last century or more (Reid 1996). As nutrition has improved, leptin concentrations may have increased with a consequent increase in growth.

While the studies reviewed above are in agreement with the present data, there is apparent disagreement between this body of evidence and the studies of Ducy *et al.* (2000). They reported that mice lacking either a functional leptin gene or the gene for its receptor had high bone densities. Both the leptin-deficient and wild-type mice showed a marked loss of bone when leptin was infused into their cerebral ventricles over a 28-day period. The key difference between these studies and those reported in the present paper is the route of delivery of the leptin. Ducy *et al.* (2000) exclusively studied the influence of central leptin administration and have produced the fascinating conclusion that there is central regulation of bone mass. This appears to be mediated by a neurological rather than an endocrine mechanism, based on preliminary reports of cross-perfusion studies (Takeda *et al.* 2000). Taken together, these findings indicate that the direct effects of leptin on bone outweigh its indirect effects mediated by the central nervous system. Since leptin originates in the periphery, including production in bone marrow adipocytes (Laharrague *et al.* 1998) and directly from mature skeletal cells themselves (Reseland *et al.* 2001, Kume *et al.* 2002), bone will be exposed to much higher concentrations of leptin than is the central nervous system. This differential is even more marked in obesity, which is associated with reduced transfer of leptin across the blood-brain barrier (Caro *et al.* 1996). Thus, *in vivo*, its direct effects on bone (anabolic and anti-resorptive) will be even more dominant than they are in the present experimental model.

The present findings of modest positive effects of leptin on bone do not mean that this is the entire explanation for the dependence of bone density on fat mass. One other mechanism involved is insulin and other factors co-secreted with it from the pancreatic beta cell (e.g.

amylin). High body weight is consistently associated with hyperinsulinaemia (Haffner & Bauer 1993, Reid *et al.* 1993) and insulin acts directly on osteoblasts *in vivo* and *in vitro* to increase cell replication and matrix synthesis (Yano *et al.* 1994, Cornish *et al.* 1996). *In vivo*, this activity is augmented by amylin, which both stimulates osteoblast growth and inhibits osteoclastic bone resorption (Cornish & Reid 1999). Insulin also acts on bone indirectly. It stimulates sex hormone production in women (Garzo & Dorrington 1984) and men (Pasquali *et al.* 1995), and it inhibits hepatic synthesis of sex hormone-binding globulin, thereby reducing protein binding of sex hormones and increasing their free concentrations (Crave *et al.* 1995, Ykijarvinen *et al.* 1995, Katsuki *et al.* 1996). Thus, hyperinsulinaemic states in humans are consistently associated with increased bone density (Haffner & Bauer 1993, Reid *et al.* 1993, Barrett-Connor & Kritzsilverstein 1996, Stolk *et al.* 1996, Wetvik 1996, Abrahamsen *et al.* 2000) and hypoinsulinaemia is associated with osteoporosis (Utsugi *et al.* 2000).

There may be a role for insulin in explaining the central effect of leptin on bone mass, since central administration of leptin dramatically decreases circulating insulin concentrations (Kamohara *et al.* 1997, Scrocchi *et al.* 1997, Shi *et al.* 1998, Tannenbaum *et al.* 1998), via changes in insulin sensitivity mediated by the autonomic nervous system (Mizuno *et al.* 1998). Conversely in the ob/ob mouse, insulin concentrations are 10-fold higher than in wild-type controls (Herberg & Kley 1975), which could contribute to its high bone mass. Systemic administration of leptin is associated with reduced insulin concentrations (Kulkarni *et al.* 1997, Scrocchi *et al.* 1997, Seufert *et al.* 1999, Shimomura *et al.* 1999), so the positive skeletal effects found in the present *in vivo* study in the face of these changes emphasise the direct effects of leptin on bone.

In conclusion, we have demonstrated that leptin acts directly on bone, influencing both osteoblasts and osteoclasts. As a result, systemic administration of leptin results in reduced bone fragility, in marked contrast to the results of its administration into the third ventricle of the brain. Since leptin is a systemic hormone, the present results imply that the peripheral effects of leptin on the skeleton outweigh its central action.

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