α-Melanocyte-stimulating hormone is a novel regulator of bone

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α-Melanocyte-stimulating hormone is a novel regulator of bone. Am J Physiol Endocrinol Metab 284: E1181–E1190, 2003. First published March 4, 2003; 10.1152/ajpendo.00412.2002.—α-Melanocyte-stimulating hormone (α-MSH), a 13-amino acid peptide derived from pro-opiomelanocortin (POMC) and produced in the brain and pituitary gland, is a key factor in the central regulation of appetite and body weight, and its production is regulated by leptin, a factor that affects bone mass when administered centrally. α-MSH acts via melanocortin receptors. Humans deficient in melanocortin receptor 4 (MC4-R) have increased bone mass, and MC4-R has been identified in an osteoblast-like cell line. Thus α-MSH may act directly on the skeleton, a question addressed by the present studies. In primary cultures of osteoblasts and chondrocytes, α-MSH dose dependently (≥10−9 M) stimulated cell proliferation. In bone marrow cultures, α-MSH (≥10−9 M) stimulated osteoclastogenesis. Systemic administration of α-MSH to mice (20 injections of 4.5 μg/day) decreased the trabecular bone volume in the proximal tibiae from 19.5 ± 1.8 to 15.2 ± 1.4% (P = 0.03) and reduced trabecular number (P = 0.001). Radiographic indexes of trabecular bone, assessed by phase-contrast X-ray imaging, confirmed the bone loss. It is concluded that α-MSH acts directly on bone, increasing bone turnover, and, when administered systemically, it decreases bone volume. The latter result may also be contributed to by α-MSH effects elsewhere, such as the adipocyte, pancreatic β-cell, or central nervous system.

Osteoblast proliferation assay ([3H]thymidine incorporation). Osteoblasts were isolated from 20-day fetal rat calvariae as previously described (13). Briefly, calvariae were excised, and the frontal and parietal bones, free of suture and periosseous tissue, were collected. The calvariae were sequentially digested using collagenase, and the cells from digests 3 and 4 were collected, pooled, and washed. Cells were grown to confluence and then subcultured into 24-well plates. Cells

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Bone Marrow Culture

Bone marrow was obtained from long bones of normal Swiss male mice aged 4–6 wk. Mice were killed by cervical dislocation while under halothane anesthesia. Femurs and tibias were aseptically removed. The bones were dissected free of adhering tissues. The epiphyses were cut off with a scalpel blade, and the marrow cavity was opened with a 23-gauge needle. The marrow cells were collected in a 50-ml centrifuge tube, spun at 1,200 rpm for 2 min, and washed with 10% fetal bovine serum (FBS)-α-MEM (both from GIBCO). Marrow cells were then cultured for 2 h in 90-mm petri dishes. After 2 h, nonadherent cells were collected, spun at 1,200 rpm for 2 min, washed with 15% FBS-α-MEM, and seeded at 10^6 cells/ml in 48-well plates (0.5 ml/well). 1.25(OH)2-vitamin D3 (1.25(OH)2D3; 10^{-8} M) was added (day 0) to all wells except negative controls, which remained in the absence of 1.25(OH)2D3 throughout the experiment. Forty-eight hours later (day 2), cultures were fed 0.5 ml of fresh medium with 1.25(OH)2D3 to make a total of 1 ml/well. After a further 48 h (day 4), cultures were fed by replacing 0.5 ml of old medium with fresh medium with 1.25(OH)2D3. Test substances were added to test groups, and vehicle was added to control groups at 0, 2, and 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, vol/vol) for 30 s. Culture plates were then stained for tartrate-resistant acid phosphatase (TRAP) by use of SIGMA Kit 387-A (Sigma Diagnostics, St Louis, MO). TRAP-positive, multinucleated cells (containing ≥3 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 1-day-old neonatal rats. The rats were killed by decapitation and the long bones aseptically removed. The bones were dissection free of adherent soft tissues. Epiphyses were removed and the remaining diaphyses split longitudinally and then placed in a tissue homogenizer containing 2 ml of acidified α-MEM (72 μl concentrated HCl/80 ml medium) with 10% FBS (GIBCO-0.6 mM MELANOCYTE-STIMULATING HORMONE AND BONE

Bone Organ Culture

Bone resorption studies were carried out in neonatal mouse calvariae as described previously (58). Mice were injected subcutaneously with 5 μCi of 45Ca at 2 days of age, and hemicalvariae were dissected out 4 days later. Hemicalvariae were preincubated for 24 h in medium 199 with 0.1% bovine serum albumin and then changed to fresh medium containing test substances or vehicle. Incubation was continued for a further 48 h. To assess DNA synthesis, [3H]thymidine (5 μCi/ml) was added in the last 4 h of the incubation. Bone was homogenized gently, and the cell suspension was collected in a 15-ml conical tube. The remaining bone tissue was placed in a small petri dish with 1 ml of 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 homogenized again in 1 ml of medium, and the suspension was once again collected. This osteoclast-rich suspension was placed onto bovine bone slices, ~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 nonosteoclastic cells, followed by a rinse in medium. The bone slices were then placed in 6-well plates (4 slices/well) containing 6 ml of medium and incubated with test substances or vehicle for 20 h. 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 cells (i.e., containing ≥3 nuclei) on each bone slice was quantified, and the cells were removed by gentle scrubbing and then stained for 30 s with toluidine blue. After numerous washes in water, the bone slices were dried and assessed for the “pits” excavated by the osteoclasts. This was achieved using reflected-light microscopy with metallurgic lenses. The results were expressed as a ratio of the number of pits to the number of osteoclasts per bone slice. There were 6–12 bone slices in each group, and each experiment was repeated two or three times.
Chondrocyte Cell Culture

Chondrocytes were isolated by removing cartilage (full-depth slices) from the tibial and femoral surfaces of adult male Swiss mice, aged between 40 and 50 days and weighing 925 ± 10 g/animal (mean ± SE). The mice were housed in a room maintained at 20°C with a light-dark cycle. They were fed Diet 86 rodent pellets (New Zealand Stockfeed) ad libitum 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. Histomorphometric analyses were carried out in the proximal tibia. The tibias were dissected free of adherent tissue, and bone lengths were recorded by measuring the distance between the proximal and the distal epiphyses with an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Tibias were then processed as previously described (11). 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 methyl methacrylate resin. Tibias were sectioned longitudinally through the frontal plane. Sections (4 µm thick) were cut using a Leitz rotary microtome and a tungsten-carbide knife and then mounted on gelatin-coated slides and air-dried. They were stained with Goldner氏's connective tissue stain and mounted on gelatin-coated slides and air-dried. They were then processed as previously described. Tibial histomorphometric analyses were made from three adjacent sections one-third of the way through the anterior/posterior depth of the proximal tibia. 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 formulas of Parfitt et al. (49). Parameters of bone formation and resorption were measured using a 20× objective in all trabecular bone tissue in the secondary spongiosa of the second of the three adjacent sections. Cell numbers were expressed per unit of bone area. Cortical width was measured on both sides of the tibial shaft 2.5 mm below the epiphysial growth plate. Epiphysial growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator (J. Cornish), who was blinded to the treatment group of each bone.

Phase-contrast X-ray imaging. X-ray microimaging was performed at CSIRO Manufacturing Science and Technology (Clayton, Victoria, Australia). This method has been described previously (72). Excised tibias from treated animals were fixed and stored in 70% alcohol. The tibias were prepared for imaging by orienting the bones in a brass clamp to enable mounting in the X-ray beam. Images were obtained using a microfocus X-ray source operated at 30 kV and with a 4-µm source size (approximate, as source is actually slightly elliptical). Samples were mounted at distance R1 = 10 cm from the X-ray source. An imaging plate was placed at distance R2 = 190 cm from the sample, resulting in an experimental magnification of ×20. Images were collected with Fuji imaging plates scanned using a BAS1000 Phosphorimager (Fuji, Japan). As described previously, imaging plates are particularly suited to the quantitative in-line phase-contrast X-ray technique because of their large linear dynamic range (29). The features of the images were quantified using MCID version 6 Elite software (Berthold, Melbourne, Australia). The trabecular region was outlined, and the circumference and length of the trabecular area were determined. A “form factor,” indicative of the shape of the trabecular area, was also calculated.

Statistical Analysis

Data are presented as means ± SE. When 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.

RESULTS

Primary Osteoblasts and Chondrocytes

α-MSH dose dependently stimulated thymidine incorporation into primary cultures of fetal rat osteoblasts, indicating a stimulation of DNA synthesis. This was accompanied by an increase in cell number after 24 h of culture. Both of these effects were seen with α-MSH concentrations of 10⁻⁸ M and greater (Fig. 1).

The effect of α-MSH on osteoblast differentiation was assessed by comparing the levels of expression of alkaline phosphatase, collagen I, and osteocalcin in osteoblasts treated with α-MSH with the expression in control cultures. Primary osteoblasts were treated with 10⁻⁸ M α-MSH, RNA was extracted after 4, 7, and 10
days of treatment, and semiquantitative RT-PCR was used to compare the levels of expression of the osteoblast differentiation markers. With this semiquantitative system, no significant differences were seen between the treatment and the control groups. Alkaline phosphatase and collagen I were expressed at high levels at all the time points studied, whereas osteocalcin was visible only on day 10 in both the treatment and the control cultures.

**Osteoclast Effects**

The effect of α-MSH on the development of osteoclasts was studied in cultures of mouse bone marrow (Fig. 3A). The number of osteoclasts present after 7 days of culture was doubled by α-MSH. In contrast, α-MSH did not affect the activity of mature osteoclasts. This was assessed from the number of resorption pits formed on bovine bone exposed for 20 h to osteoclasts prepared from neonatal rats (Fig. 3B). These results were confirmed in studies of a second model of mature osteoclast function, the neonatal mouse calvaria (Fig. 3C). Because there is virtually no bone marrow in this tissue, these explants reflect mature osteoclast function and again showed no effect of α-MSH treatment.

**Systemic Administration of α-MSH**

Normal adult male mice were treated systemically with α-MSH or vehicle over a 4-wk period. Histomorphometry of the proximal tibias indicated that bone turnover was increased in the α-MSH-treated animals (osteoblast index: control 20.1 ± 1.5, α-MSH 28.6 ± 1.7, *P* < 0.0007; osteoclast index: control 0.26 ± 0.02, α-MSH 0.29 ± 0.03, not significant (NS)). However, trabecular bone volume was decreased by almost one-quarter in the animals receiving α-MSH (Fig. 4). This resulted from a decrease in trabecular number and an increase in trabecular separation rather than from any change in trabecular thickness (control 24.5 ± 1.3, α-MSH 25.1 ± 1.1 μm, *P* = 0.74). There was no difference in cortical width between the groups (control 0.19 ± 0.01, α-MSH 0.19 ± 0.01 mm), and growth plate thickness was also comparable in the two groups (control 0.087 ± 0.004, α-MSH 0.087 ± 0.004 mm).

The results of the histomorphometric analysis of trabecular structure were confirmed by phase-contrast X-ray images of the tibias. Figure 5A shows images of three tibias from the control group and three from the α-MSH-treated group. Simple inspection indicates that the extent of trabecular bone is diminished following α-MSH treatment. In the α-MSH group, the perimeter

**Fig. 1.** Effect of α-melanocyte-stimulating hormone (α-MSH) on cell number and thymidine incorporation in primary cultures of fetal rat osteoblasts at 24 h. *Significantly different from control, *P* ≤ 0.04; **significantly different from control, *P* ≤ 0.01.

**Fig. 2.** Effect of α-MSH on cell number and thymidine incorporation in primary cultures of canine chondrocytes at 24 h. *Significantly different from control, *P* ≤ 0.05.
and length of the trabecular area were decreased by 34 (P < 0.0005) and 32% (P < 0.002), respectively (Fig. 5B). The form factor of the trabecular zone was unchanged. These results indicate that α-MSH caused a reduction in trabecular bone mass.

Weight gains during the study were not significantly different in the two groups; weight increased in the course of the study from 27.5 ± 0.8 to 33.6 ± 0.8 g in animals treated with vehicle and from 27.4 ± 0.8 to 33.2 ± 0.5 g in those receiving α-MSH. However, the percent fat mass of the animals at the end of the experiment was lower in the α-MSH-treated group (8.7 ± 0.6%) compared with the control group (10.2 ± 0.5%, P = 0.05). Tibial lengths were not different between the groups (control 18.6 ± 0.1, α-MSH 18.7 ± 0.1 mm).

**DISCUSSION**

The present study is the first to address the direct effects of α-MSH on skeletal cells. It demonstrates that this peptide increases the proliferation of osteoblasts without affecting their differentiation, increases chondrocyte proliferation, and stimulates the development

**Fig. 3.** A: effect of α-MSH on osteoclast development in mouse bone marrow cultures. The number of multinucleated (MNC) cells staining positively (+ve) for tartrate-resistant acid phosphatase (TRAP) was significantly increased by α-MSH (P < 0.03). Salmon calcitonin (sCT) was included as a positive control and significantly reduced osteoclast numbers (P < 0.01). B: effect of α-MSH on pit formation on bovine bone by isolated mature rat osteoclasts. No significant effects were detected. C: effect of α-MSH on calcium release from cultured neonatal mouse calvariae. There were no significant effects.

**Fig. 4.** Effect of daily administration of α-MSH (20 times over 4 wk) on trabecular bone volume, trabecular number, and trabecular separation in the proximal tibias of normal adult male mice. *Significantly different from control, P = 0.03; **significantly different from control, P < 0.01.
of osteoclasts from their precursors in bone marrow. However, α-MSH does not act directly on the mature osteoclast either when studied in isolated cell culture or when assessed in an organ culture model lacking osteoclast precursors. Although α-MSH is a neuropeptide, it does have direct actions on cells outside the central nervous system (3). As its name implies, it regulates pigment production in melanocytes (44, 60), and there is also evidence of direct actions of α-MSH on adipocytes [where it promotes lipolysis (4)] and the pancreatic β-cell [where it decreases insulin secretion (64)]. Its adipocyte effects are particularly relevant to the present study, since osteoblasts and adipocytes share a common precursor in the bone marrow stromal cell. The concentrations of α-MSH found to be effective in skeletal cells in the present studies are comparable to those that are active in in vitro studies of adipocytes (4) and pancreatic β-cells (64) and are consistent with the EC50 values for cAMP production stimulated by α-MSH via MC1-R, MC3-R, or MC4-R (~10^{-9} M) in transfected cell lines (26, 27, 43, 45, 61). The lowest active concentrations of α-MSH in osteoblasts and chondrocytes are comparable to the circulating concentrations in humans (34, 47) and rats (69), suggesting that these direct actions in bone may be physiologically relevant.

The results of systemic administration of α-MSH to adult mice are generally consistent with the in vitro findings in that increased bone turnover was observed in both models. In vivo, this was significant with respect to the histomorphometric assessment of osteoblast numbers but not those of osteoclasts, possibly reflecting the inaccuracy of the assessment of bone resorption indexes from static histomorphometry. However, the balance of these effects was toward bone loss, resulting in a 22% decrease in trabecular bone volume in the animals receiving α-MSH and similar changes in the radiographic indexes of trabecular mass. It would be of interest to extend these studies to a wider range of doses of α-MSH and its analogs to determine whether the balance of the opposing effects on osteoblasts and osteoclasts is affected by either of these variables. The present findings suggest that, in vivo, the stimulatory effects of α-MSH on osteoclastogenesis predominate over its actions on osteoblast development, although the histomorphometry does not provide clear evidence of this. A second explanation is that the direct effects of α-MSH on bone cells are

Fig. 5. A: phase-contrast X-ray images of the trabecular region of tibias from control (left) and α-MSH-treated mice (right). B: perimeter and length of the trabecular regions were quantified as described in METHODS and were significantly reduced in the animals treated with α-MSH, *p < 0.0005, **p < 0.002.
modulated by its effects on other tissues such as adipose tissue, the pancreatic β-cell, or the central nervous system. These possibilities will now be discussed.

As reviewed above, fat mass and bone mass have frequently been found to be positively related to one another (35, 51, 53, 55, 59), so the lower fat mass of the α-MSH-treated animals is likely to contribute to the negative effect of α-MSH on bone mass. This may have been contributed to, in part, by the lower circulating leptin levels following treatment with α-MSH (9, 23). The effect of α-MSH on fat mass is well established: from studies of α-MSH administration in humans (23), from studies in mice (32) and humans (22, 28, 31, 70) lacking a functioning MC4-R, from findings in mice and humans deficient in the α-MSH-precursor peptide POMC (37, 74), and in the agouti mouse, which over-expresses an antagonist of MC4-R (32). The weight-regulatory actions of centrally administered α-MSH are the product of appetite suppression and increased metabolism resulting from α-MSH effects in the hypothalamus (6), although there may also be some contribution from its direct lipolytic effects on adipocytes (4). When the peptide is administered peripherally, as in the present studies, effects on fat mass presumably result from the same two mechanisms of action, although the central effect must be considerably less.

α-MSH has been shown to directly inhibit secretion of insulin from the pancreatic β-cell (64), and decreased circulating insulin concentrations have also been reported in humans treated systemically with α-MSH analogs (23). In vivo, insulin concentrations correlate closely with bone density (57, 66), and this relationship may have a number of mechanisms. Insulin interacts directly with osteoblasts, stimulating cell proliferation in vitro and in vivo (14, 30, 52). Also, insulin inhibits sex hormone-binding globulin production in the liver, thereby increasing free concentrations of sex hormones (25, 39, 50). Furthermore, insulin is cosecreted with other bone-active factors such as amylin (a direct stimulator of osteoblast growth and an inhibitor of osteoclasts (10)), and preptin, a fragment of pro-IGF-II, (5) is also a potent osteoblast growth factor (8). Thus a cascade of bone-anabolic factors comes from the pancreatic β-cell and will be reduced by α-MSH treatment. Blockade of this pathway may contribute to the bone loss seen in the in vivo study.

Systemically administered α-MSH is most likely acting primarily through peripheral melanocortin receptors, although there is evidence that it may cross the blood-brain barrier to a small extent (15), so a central mechanism of action cannot be completely ruled out. The concept of a centrally acting agent impacting on bone was first introduced by Ducy et al. (16), who demonstrated that the intracerebroventricular injection of the adipocyte hormone leptin resulted in trabecular bone loss similar to that observed in the present in vivo study. The present findings may contribute to an explanation of the work of Ducy et al., since α-MSH is an important mediator of the central effects of leptin. Leptin action in the hypothalamus stimulates α-MSH release from neurons of the arcuate nucleus (63), resulting in appetite suppression and weight loss and leading to effects mediated by the autonomic nervous system. These include the regulation of insulin secretion, which is decreased by as much as 80% by central α-MSH administration (20). This effect is abrogated by sympathetic blockade with phentolamine (20), confirming that it is mediated by the autonomic nervous system. Thus any central effects of α-MSH in this model may act in concert with its direct effects on the pancreatic β-cell, described above, to decrease bone mass.

Takeda et al. (68) have recently provided significant new information regarding the relationship between the bone effects of leptin and α-MSH. They reported that the intracerebroventricular administration of an α-MSH analog to ob/ob mice does not affect bone mass; that the yellow agouti mouse, which is resistant to α-MSH, has a normal bone mass and shows the same bone response to leptin as wild-type mice; and that mice deficient in MC4-R have normal bone mass. However, the latter finding directly contradicts the report of Farooqi et al. (22) in humans with this deficiency, in whom bone mass is markedly increased. This discrepancy could be related to differences between species in the role of α-MSH, differences in the gene mutation, effects of the mutations on skeletal size, or differences in techniques for assessment of bone mass (histomorphometry of trabecular bone in the vertebral body in the mouse studies vs. whole body dual-energy X-ray absorptiometry in the humans). Confirmation of the results of Takeda et al. will be important to clarify understanding in this area. In the meantime, the present data tend to support the suggestion from the study of Farooqi et al. that α-MSH does have an important effect on bone mass.

There is a striking parallel in the present data between the effects of α-MSH on chondrocytes and osteoblasts. α-MSH is a potent mitogen for both cell types in vitro, but there is no evidence of this effect in vivo. In the case of chondrocytes, this is reflected in the lack of effect of α-MSH on the width of the growth plate or on tibial growth. This contrasts with our previous work in this mouse model, in which factors stimulating chondrocyte proliferation to a degree comparable to that seen with α-MSH have also increased growth plate width and tibial length in vivo (7, 12). It is likely that the catabolic effects of systemically administered α-MSH mediated by the hormonal and neuronal mechanisms discussed in the three preceding paragraphs also account for this dissociation of its in vitro and in vivo effects on cartilage.

This is not the first study of the effects of systemic α-MSH administration on bone. Stenstrom et al. (65) showed that α-MSH administration for 20 days increased cortical width in hypophysectomized rats. In contrast, Aspenberg et al. (2) found no effect in similar studies in normal rats, implying that their result was specific to hormone-deficient animals. Thus both of these results agree with those of the present study, which also found no effect on cortical bone. Neither of these studies assessed trabecular bone. An ACTH analog has been shown to either stimulate or depress
osteoblast proliferation in vivo, depending on dose and time of day (71). The variability of these effects probably reflects an interaction with endogenous ACTH release, which itself shows a marked diurnal rhythm. Interference with secretion of endogenous peptides is another mechanism by which the effects of α-MSH on bone may be modified in vivo.

In conclusion, α-MSH acts directly on skeletal cells to stimulate their proliferation, but in vivo it leads to a net loss of trabecular bone. This may be mediated, to some extent, by the effects of α-MSH to decrease fat mass and to diminish the secretion of anabolic hormones from the pancreatic β-cell. It may contribute to the bone loss that follows from the intracerebroventricular administration of leptin. If α-MSH analogs were to be developed for the treatment of obesity, then monitoring of their effects on bone would be an important safety consideration.

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