

The GH secretagogues ipamorelin and GH-releasing peptide-6 increase bone mineral content in adult female rats

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Abstract

Growth hormone (GH) is of importance for normal bone remodelling. A recent clinical study demonstrated that MK-677, a member of a class of GH secretagogues (GHSs), increases serum concentrations of biochemical markers of bone formation and bone resorption. The aim of the present study was to investigate whether the GHSs, ipamorelin (IPA) and GH-releasing peptide-6 (GHRP-6), increase bone mineral content (BMC) in young adult female rats. Thirteen-week-old female Sprague–Dawley rats were given IPA (0.5 mg/kg per day; $n=7$), GHRP-6 (0.5 mg/kg per day; $n=8$), GH (3.5 mg/kg per day; $n=7$), or vehicle administered continuously s.c. via osmotic minipumps for 12 weeks. The animals were followed *in vivo* by dual X-ray absorptiometry (DXA) measurements every 4th week. After the animals were killed, femurs were analysed *in vitro* by mid-diaphyseal peripheral quantitative computed tomography (pQCT) scans. After this, excised femurs and vertebrae L6 were analysed by the use of Archimedes' principle and by determinations of ash weights. All treatments increased body weight and total tibial and vertebral BMC measured by DXA *in vivo*

compared with vehicle-treated controls. However, total BMC corrected for the increase in body weight (total BMC:body weight ratio) was unaffected. Tibial area bone mineral density (BMD, BMC/area) was increased, but total and vertebral area BMDs were unchanged. The pQCT measurements *in vitro* revealed that the increase in the cortical BMC was due to an increased cross-sectional bone area, whereas the cortical volumetric BMD was unchanged. Femur and vertebra L6 volumes were increased but no effect was seen on the volumetric BMDs as measured by Archimedes' principle. Ash weight was increased by all treatments, but the mineral concentration was unchanged. We conclude that treatment of adult female rats with the GHSs ipamorelin and GHRP-6 increases BMC as measured by DXA *in vivo*. The results of *in vitro* measurements using pQCT and Archimedes' principle, in addition to ash weight determinations, show that the increases in cortical and total BMC were due to an increased growth of the bones with increased bone dimensions, whereas the volumetric BMD was unchanged.

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Introduction

Both clinical and animal studies have demonstrated that growth hormone (GH) is important for normal bone modelling and remodelling (Bouillon 1991, Eriksen *et al.* 1996, Ohlsson *et al.* 1998). In young adult rats, GH treatment increases bone mass by increased longitudinal growth at the growth plate and by increased subperiosteal bone deposition (Ohlsson *et al.* 1998). In old male rats, 80 days of GH treatment increased cortical bone formation mainly through increased subperiosteal bone formation (Andreassen *et al.* 1995). In young and in old rats, GH treatment increased cortical mechanical strength, mainly as a result of an increase in bone dimensions (Jørgensen *et al.*

1991, Andreassen *et al.* 1995). In primates, GH but not insulin-like growth factor (IGF)-I given to female monkeys for 7 weeks increased bone formation as measured with mineral apposition rate, and the bone formation rate (Sass *et al.* 1997). In GH-deficient (GHD) humans, long-term GH treatment increased total bone mineral content (BMC) and area bone mineral density (BMD) in several weight-bearing skeletal locations (Johannsson *et al.* 1996). Studies to date have not shown any impressive effect of GH on bone mass in non-GHD subjects.

The mechanism for the stimulatory effect of GH on the amount of bone is not fully understood. Normal bone metabolism is regulated by a balance between bone formation caused by osteoblasts and bone resorption caused

by osteoclasts. Osteoblastic cells, in addition to being directly responsible for bone formation, control bone resorption by regulating the proliferation, differentiation and recruitment of osteoclast progenitors (Takahashi *et al.* 1988). Human osteoblast-like cells express functional GH receptors and GH has been shown to exert an anabolic effect on the osteoblasts (Kassem *et al.* 1993, Nilsson *et al.* 1995). These findings indicate a direct stimulatory effect of GH on bone formation.

Pituitary GH secretion is regulated by the stimulatory peptide GH-releasing hormone (GHRH) and the inhibitory peptide somatostatin (Frohman & Jansson 1986). The new class of synthetic GH secretagogues (GHSs) that has been developed includes GH-releasing peptides (GHRPs) such as GHRP-2, GHRP-6 and hexarelin, and non-peptidyl oral GHSs such as MK-677 (Smith *et al.* 1998). A G protein-coupled receptor has been found to be a target for these substances (Howard *et al.* 1996). An endogenous ligand specific for the GHS receptor was recently described (Kojima *et al.* 1999). At the hypothalamic level, the GHSs stimulate GH secretion by activating arcuate nucleus GHRH neurones (Dickson & Luckman 1997), resulting in increased release of GHRH into portal blood (Guillaume *et al.* 1994). The GHSs also stimulate GH secretion by a direct action at the pituitary level (Bowers *et al.* 1984), where the GHSs may act as functional antagonists of somatostatin action (Smith *et al.* 1998). The stimulatory effect on GH release seems to be persistent, as the GHRPs hexarelin and GHRP-2 have been reported to increase growth velocity in children with short stature/GHD (Laron *et al.* 1995, Mericq *et al.* 1998). In addition to GH release, most GHSs have been found to elicit a transient cortisol response (Svensson *et al.* 1998a). However, the novel GHS, ipamorelin, has not yet been found to stimulate glucocorticoid release (Raun *et al.* 1998).

The GHSs enhance the amplitude of the physiological GH pulses (Smith *et al.* 1998), whereas an s.c. injection of GH induces a bell-shaped curve of serum GH concentrations that are continuously high for more than 12 h (Oscarsson *et al.* 1997). Little is known about the effects on bone of an increase in the amplitude of endogenous pulsatile GH secretion. We have recently shown that short-term treatment of obese men with the oral GHS, MK-677, increases circulating biochemical markers of bone formation and bone resorption (Svensson *et al.* 1998b). The aim of the present study was to determine the effect of 12 weeks of treatment with the GHSs ipamorelin and GHRP-6 on bone mass in young adult female Sprague–Dawley rats.

Material and Methods

Animals

Female Sprague–Dawley rats ($n=30$; 13 weeks old, mean body weight 228 g at onset; obtained from BK Universal,

Stockholm, Sweden) were housed under standardised environmental conditions (24–26 °C, 50–60% relative humidity, artificial lighting on from 0500 to 1900 h). The rats were given free access to water and to standard rat chow containing 0.8% calcium and 0.6% phosphorus (BK Universal). All experimental procedures were carried out according to ethical guidelines for animal experimentation at the University of Göteborg.

Study procedure

Over a 12-week period, rats were infused s.c. with human GH (3.5 mg/kg per day; Norditropin; Novo Nordisk A/S, Copenhagen, Denmark; $n=7$), GHRP-6 (0.5 mg/kg per day, Bachem, Bubendorf, Switzerland; $n=8$), ipamorelin (0.5 mg/kg per day; Novo Nordisk A/S; $n=7$) or vehicle (0.05% acetic acid in sterile 0.5% saline; $n=8$) using osmotic minipumps (Alzet 2004; Alza Corp., Palo Alto, CA, USA). *In vivo* dual X-ray absorptiometry (DXA) measurements were performed at baseline and after 4, 8 and 12 weeks of treatment. Before the DXA measurements, the rats were anaesthetised with a combination of ketamine (1.8 ml/kg; Ketalar, Park Davies, Morris Plains, NJ, USA; 67 mg/kg) and xylazine (13 mg/kg; Rompun, Bayer AG, Leverkusen, Germany) given i.p. During anaesthesia, the osmotic minipumps were removed and body weight and DXA measurements were performed, followed by implantation of new osmotic minipumps. Food intake was measured at baseline and after 4, 8 and 12 weeks of treatment by the use of metabolic cages. At the end of the experiment, rats were killed by decapitation and excised bones were stored in ethanol, then excised femurs were analysed by using peripheral quantitative computed tomography (pQCT). After this, femurs and vertebrae L6 were analysed by the use of Archimedes' principle and by determinations of ash weights.

Dual X-ray absorptiometry

BMC (in g) and area BMD (in g/cm²) were measured *in vivo* by DXA (pDEXA Sabre; Norland Corp., Fort Atkinson, WI, USA). The software Sabre Research (version 3.6) was used. Total body BMC and area BMD (BMC/area) were measured from the lower level of thoracic vertebra 9 to the inferior level of the first tail vertebra (the maximal scan area of the DXA). BMC and area BMD of tibia were measured from the knee joint to the joint between tibia and calcaneus (and thus these measurements also include fibula). BMC and area BMD of vertebra were measured from the superior level of lumbar vertebra 3 to the inferior level of lumbar vertebra 6 (L3–L6). The interassay coefficients of variation (CVs) for the DXA measurements were less than 4%.

Femur length and vertebral body L6 height

The length of femurs was determined *in vitro* with DXA. The femurs were placed in an anatomical position (facing

Table 1 Body weight and bone dimensions after 12 weeks of treatment of female Sprague–Dawley rats with IPA, GHRP-6, GH or vehicle. All values are presented as the mean (S.E.M.)

Variable	Vehicle (n=8)	IPA (n=7)	GHRP-6 (n=8)	GH (n=7)
Body weight at baseline (g)	234 (8)	223 (9)	230 (8)	223 (8)
Body weight after 12 weeks of treatment (g)	320 (8)	340 (9)**	354 (5)**	338 (5)**
Body weight increase (%)	37.6 (3.5)	52.9 (5.9)**	55.1 (4.9)**	53.3 (5.8)**
Femur length (mm)	37.2 (0.4)	37.9 (0.2)	38.4 (0.2)*	37.8 (0.2)
Vertebral body L6 height (mm)	7.67 (0.07)	7.82 (0.10)	7.85 (0.05)	7.74 (0.08)

For body weight after 12 weeks of treatment, *P*-values (compared with vehicle) were calculated by two-way ANOVA of the percent change from baseline, followed by Student–Newman–Keuls *post hoc* test. *P* values for differences in femur length and vertebral body L6 height were calculated by one-way ANOVA followed by Student–Newman–Keuls *post hoc* test.

P*<0.05, *P*<0.01, compared with vehicle.

anteriorly) in a Petri dish. The length of each femur was determined by lining up the cursors on the screen with the trochanter at the top and the lateral condyle at the bottom of the femur. The distance between the cursors was measured by a ruler tool in the software. The CV was less than 1%.

The body of vertebra L6 was cleaned of all soft tissue, including the intravertebral discs. Then the height of vertebral body L6 was determined at the middle of the anterior surface using a micrometer. The CV was less than 1%.

Peripheral quantitative computed tomography

Tomographic measurements were performed using the Stratec pQCT XCT Research M (Norland Corp.) specifically modified for use on small bone specimens (software version 5.4B; resolution 70 μ m (Windahl *et al.* 1999)). Cortical volumetric BMD (BMC/volume), the cortical cross-sectional area and cortical BMC were determined by mid-diaphyseal pQCT scans of excised femurs. The inter-assay CVs for the pQCT measurements were less than 3%.

Archimedes' principle and ash weights

The whole femurs and vertebrae were immersed in distilled water and submitted to vacuum for at least 10 min to remove trapped air bubbles. Then the wet weight and the weight of the bones in distilled water were determined. After the bones had been defatted in acetone for 2 days followed by freeze-drying for 3 days, the dry defatted weight was determined (Sandstedt *et al.* 1996). The (real) volume was calculated as (dry defatted weight – weight in distilled water)/density of distilled water. The (real) volumetric BMD was calculated as dry defatted weight/(real) volume of the bone. The ash weight (mineral weight) was determined after incineration in a muffle oven at 105 °C

for 2 h, followed by 580 °C for 24 h. The mineral concentration was calculated by dividing mineral weight by dry defatted weight.

Metabolic cages

The rats were housed for 2 days in metabolic cages at baseline and after 4, 8 and 12 weeks of treatment (before the DXA measurements at each time point). Water and powdered food were freely available and food intake was measured daily.

Statistics

All the descriptive statistical results that are presented are given as the arithmetic mean and standard error of the mean (S.E.M.). Bartlett's test was performed to evaluate if data were normally distributed. For skewedly distributed data (cortical BMD, cross-sectional cortical bone area and cortical BMC obtained from the pQCT measurements of femurs *in vitro*), a logarithmic transformation was performed before the analysis of between-group differences. For body weight and for data obtained from DXA measurements *in vivo*, a two-way analysis of variance (ANOVA) of the percent change from baseline were performed with time and treatment as the independent variables, followed by Student–Newman–Keuls *post hoc* test. The data obtained from *in vitro* measurements (pQCT, Archimedes' principle, ash weight) were analysed by a one-way ANOVA with treatment as the independent variable, followed by Student–Newman–Keuls *post hoc* test. A two-tailed *P*<0.05 was considered significant.

Results

Body weight, food intake and dimensions of bones

Baseline body weight was similar in all study groups. However, the increase in body weight was larger after all

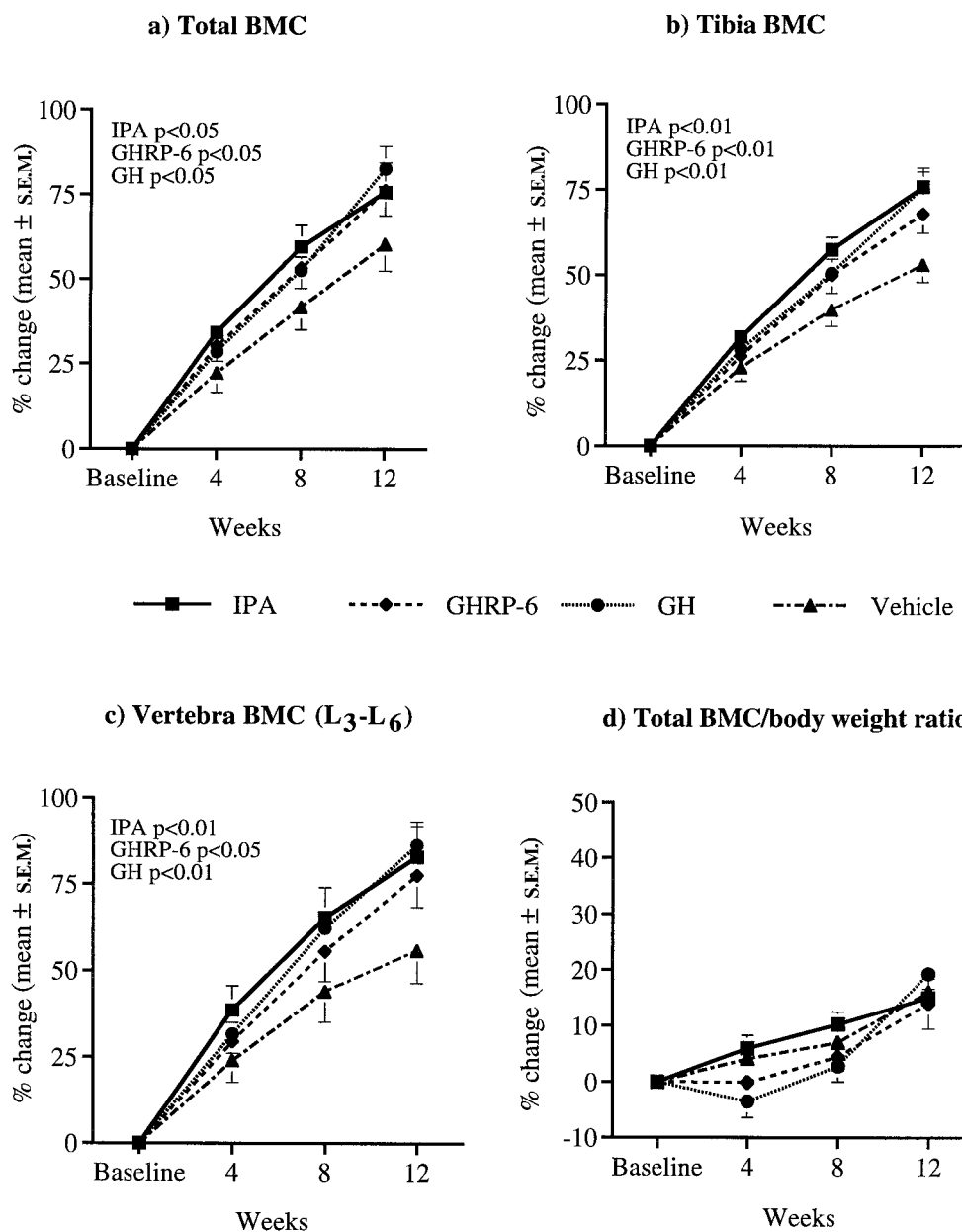


Figure 1 Percentage change in (a) total body BMC, (b) tibia BMC, (c) vertebral BMC (L₃-L₆), and (d) total BMC/body weight ratio during 12 weeks of treatment of female Sprague-Dawley rats with IPA, GHRP-6, GH or vehicle. The BMC values were measured by DXA *in vivo*. The *P* values shown (compared with vehicle) were calculated by two-way ANOVA of the percent change from baseline, followed by Student-Newman-Keuls *post hoc* test.

treatments than after vehicle (Table 1). Food intake was unchanged (data not shown). Femur length was increased in the GHRP-6-treated rats compared with vehicle-treated rats, but that in the GH- and IPA-treated rats was not statistically changed (Table 1). The height of vertebral body L6 was unchanged (Table 1).

DXA measurements of BMC *in vivo*

There were no differences at baseline in any of the bone parameters measured by DXA (data not shown). As measured by DXA *in vivo*, both of the GHSs (IPA and GHRP-6) and GH increased total, tibial and vertebral BMCs compared with vehicle (Fig. 1a-c). However,

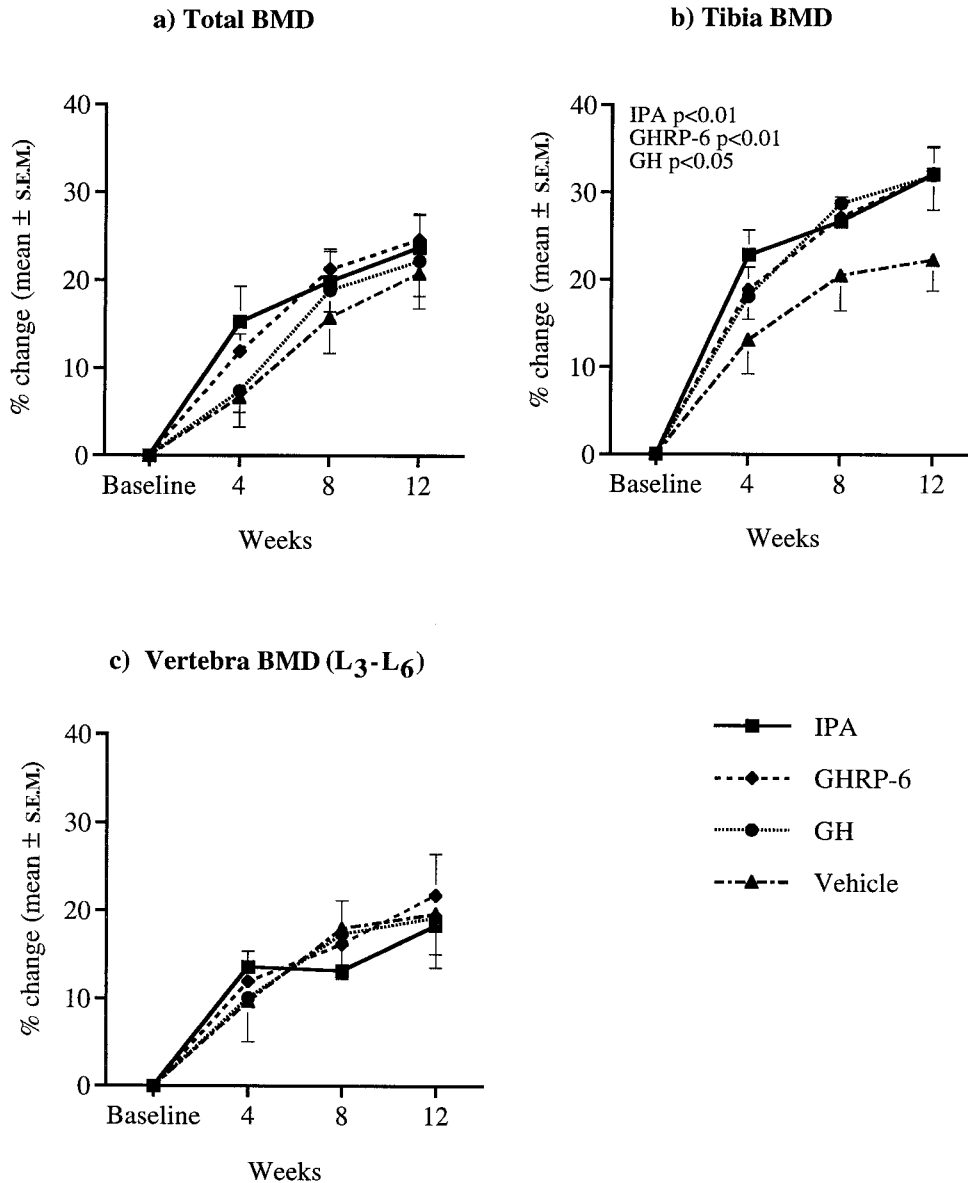


Figure 2 Percent change in (a) total body BMD, (b) tibia BMD, and (c) vertebral BMD (L₃-L₆) as measured by DXA *in vivo* during 12 weeks of treatment of female Sprague-Dawley rats with IPA, GHRP-6, GH or vehicle. No effect was found on total or vertebral BMD, but all treatments induced a significant increase in tibia BMD compared with the vehicle-treated controls. The *P* values shown (compared with vehicle) were calculated by two-way ANOVA of the percent change from baseline, followed by Student-Newman-Keuls *post hoc* test.

when the increase in total BMC was corrected for the increase in body weight (total BMC/body weight ratio), the increase in total BMC lost statistical significance (Fig. 1d). Spine and tibia BMC also were unchanged when corrected for body weight (data not shown).

All substances increased the tibial area BMD, as measured by DXA, but no significant effect was seen on total and vertebral area BMDs (Fig. 2a-c).

Effects on cortical bone as measured using pQCT

Mid-diaphyseal pQCT scans of excised right femurs demonstrated that the cortical BMC was increased in the IPA and GH groups (IPA $P < 0.05$ compared with vehicle; GH $P < 0.05$ compared with vehicle; GHRP-6 $P = 0.06$ compared with vehicle; Table 2). The increase in mid-diaphyseal cortical BMC was due to an increase in

Table 2 Cortical BMD, cross-sectional cortical bone area and cortical BMC, as measured by mid-diaphyseal pQCT scans of femurs *in vitro*, after 12 weeks of treatment of female Sprague–Dawley rats with IPA, GHRP-6, GH or vehicle. All values are presented as the mean (S.E.M.)

Variable	Vehicle (n=8)	IPA (n=7)	GHRP-6 (n=8)	GH (n=7)
Cortical BMD (mg/mm ³)	1.355 (0.004)	1.355 (0.004)	1.348 (0.004)	1.357 (0.004)
Cross-sectional cortical bone area (mm ²)	5.50 (0.15)	5.92 (0.09)*	5.80 (0.11)*	5.92 (0.07)*
Cortical BMC (mg/mm)	7.46 (0.22)	8.02 (0.12)*	7.82 (0.16)**	8.04 (0.09)*

Between-group differences were calculated by one-way ANOVA followed by Student–Newman–Keuls *post hoc* test.
P*<0.05, *P*=0.06, compared with vehicle.

Table 3 Results of *in vitro* measurements using Archimedes' principle and ash weight determinations after 12 weeks of treatment of female Sprague–Dawley rats with IPA, GHRP-6, GH or vehicle. All values are presented as the mean (S.E.M.)

Variable	Vehicle (n=8)	IPA (n=7)	GHRP-6 (n=8)	GH (n=7)
Dry defatted weight (mg)				
Femur	580.2 (13.8)	624.0 (9.9)*	613.8 (7.7)*	605.2 (7.9)*
Vertebra L6	191.8 (5.4)	216.4 (3.8)**	207.2 (3.0)*	203.4 (3.0)*
Bone volume (mm ³)				
Femur	255.8 (5.6)	274.8 (4.4)*	268.4 (3.7)*	270.4 (3.8)*
Vertebra L6	86.1 (2.5)	99.1 (1.8)**	94.8 (1.6)**	94.4 (1.8)**
Volumetric BMD (g/cm ³)				
Femur	2.27 (0.01)	2.27 (0.01)	2.29 (0.01)	2.24 (0.02)
Vertebra L6	2.23 (0.02)	2.18 (0.02)	2.19 (0.02)	2.16 (0.03)
Ash weight (mg)				
Femur	407.7 (8.4)	437.1 (4.5)**	430.6 (4.7)*	427.0 (4.3)*
Vertebra L6	131.2 (3.3)	148.3 (2.5)**	143.9 (2.7)**	141.6 (2.3)**
Mineral concentration†				
Femur	70.3 (0.3)	70.1 (0.8)	70.2 (0.2)	70.6 (0.4)
Vertebra L6	68.5 (0.5)	68.6 (0.7)	69.4 (0.8)	69.6 (0.6)

Between-group differences were calculated by one-way ANOVA followed by Student–Newman–Keuls *post hoc* test.

†Ash weight/dry defatted weight.

P*<0.05, *P*<0.01, compared with vehicle.

cross-sectional cortical bone area, whereas the cortical volumetric BMD was unaffected (Table 2).

Archimedes' principle and ash weight

The dry defatted weight of femur and vertebra L6 was increased by all treatments compared with the vehicle-treated controls (Table 3). Measurements using Archimedes' principle showed that the volumes of femur and vertebra L6 were increased, but the volumetric BMD was unchanged (Table 3). Ash weight was increased for all treatments, but the mineral concentration, determined as ash weight per dry defatted weight, was unchanged (Table 3).

Discussion

The present study demonstrates that treatment with the GHSs, ipamorelin and GHRP-6, increases BMC in

normal adult female rats. The results of several different measurements clearly indicate that the increases in cortical and total BMC were caused by increased bone volume, whereas the volumetric BMD was unchanged. The pQCT measurements of femurs *in vitro* showed an increase in cortical BMC that was due to an increase in the cross sectional bone area. Femur and vertebra L6 volumes were increased as measured by Archimedes' principle, whereas no effect was seen on the volumetric BMDs. Finally, ash weight (i.e. mineral content) was increased in all treatment groups, whereas the mineral concentration was unchanged.

In the present study, treatment with the GHSs and with GH itself increased body weight compared with vehicle, suggesting that the doses used and the route of administration of treatments were effective. The sustained increase in body weight in the GH-treated rats made it possible to rule out the possibility of species-dependent development of active antibodies against the human GH. This finding is supported by several previous studies

demonstrating a sustained effect of human GH on BMC in rats (Andreassen *et al.* 1995, 1996). A desensitisation of the initial GH response has previously been observed after repeated GHS administration (Smith *et al.* 1998). In contrast, in the present study, a sustained increase in body weight and BMC was seen for IPA and GHRP-6, indicating that GHSs can also exert stimulatory effects in treatment prolonged over several months.

Total food intake was unaffected by both GHS and GH treatment in the present study. An acute increase in food intake has been observed after intracerebroventricular administration of GHSs (Locke *et al.* 1995, Okada *et al.* 1996), but in this study, as in a previous clinical study (Svensson *et al.* 1998a), no effect has been observed after prolonged systemic or oral administration of GHSs. GH administration is known to increase food efficiency in rats (weight gain/food weight (Verrat-Durebex *et al.* 1999)). Therefore, increased food efficiency due to increased circulating GH is a likely explanation for the increases in body weight induced by all treatments in this study.

GH treatment and GHS treatment increased tibia area BMD as measured by DXA *in vivo* in the present study. However, the pQCT measurements of femurs *in vitro* revealed an unaffected cortical volumetric BMD and the measurements using Archimedes' principle showed an unchanged total femur and vertebra L6 volumetric BMD. Other studies have also shown that long-term GH treatment increases area BMD as measured by DXA (Yeh *et al.* 1994, Ohlsson *et al.* 1998), but the volumetric BMD (BMC/volume), as measured by Archimedes' principle or pQCT (Jørgensen *et al.* 1991, Andreassen *et al.* 1995, Rosen *et al.* 1995), is unchanged. These findings could be explained by an overestimation of the increase in BMD by DXA. Area BMD (BMC/area) does not account for the bone thickness perpendicular to the two-dimensional DXA image. Therefore, when cortical bone mass is increased because of enhanced subperiosteal bone deposition, this itself may result in enhanced area BMD as measured by DXA.

The increases in BMCs were of a magnitude similar to that in body weight. In almost all studies in rats, GH administration has resulted in increased body weight (Ohlsson *et al.* 1998). It can not be excluded that the increase in BMC is due to increased mechanical load exerted on the skeleton by the increased body weight. However, administration of GH or IGF-I also increased subperiosteal bone formation under weightless conditions during space flight, and the amount of added bone was similar to that obtained by GH or IGF-I administration on the ground (Turner 1995, Bateman *et al.* 1998). Therefore, the present increase in bone dimensions is likely to be due to a direct effect of GH on total bone growth. This may be caused by a radial bone growth resulting from increased subperiosteal bone formation. GH has been shown to increase subperiosteal bone deposition in both young and old adult rats (Andreassen *et al.* 1995,

Ohlsson *et al.* 1998), and an increased cross-sectional cortical bone area was also seen in this study.

The present increase in bone volume may also have been due to linear bone growth derived from the growth plate. In rats, the growth plates first begin to close when the rats are very old (Ohlsson *et al.* 1998). For this reason, most studies have also found a small stimulatory effect on longitudinal growth in adult rats (Ohlsson *et al.* 1998). Therefore, it is likely that linear bone growth was also stimulated in our present study. The measurements of femur length and vertebral body L6 height only showed a significant increase for femur height in the GHRP-6-treated rats. However, small stimulatory effects on linear bone growth may not have been detected statistically in the GH- and ipamorelin-treated groups, as a result of the study design (seven or eight rats in each group).

GH administered by osmotic minipumps induces a continuous increase in circulating GH concentrations, whereas the GHSs, also when administered continuously, increase the amplitude of the endogenous GH pulses (Huhn *et al.* 1993, Jaffe *et al.* 1993). Furthermore, it has been suggested that different subtypes of the recently cloned GHS receptor may exist and that different GHSs may differ in their binding affinity for the different subtypes (Ong *et al.* 1998). Despite these differences, the effects on body growth and BMC were similar for all treatments with the doses and the route of administration used in the present study. There are also non-peptidyl GHSs that produce a potent GH response after oral administration (Ankersen *et al.* 1998, Hansen *et al.* 1998, Smith *et al.* 1998). Further studies are needed to investigate whether orally administered non-peptidyl GHSs also increase BMC.

In a previous clinical study we found that a 2-month treatment with the GHS, MK-677, increases circulating markers of bone formation and resorption (Svensson *et al.* 1998b). We have now shown that treatment of female rats with the GHSs ipamorelin and GHRP-6 for 3 months increased BMC as measured by DXA *in vivo*. The results of *in vitro* measurements using pQCT and Archimedes' principle, in addition to the ash weight determinations, show that the increases in cortical and total BMC were due to an increased growth of the bones with increased bone dimensions, the volumetric BMD remaining unchanged.

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