Ghrelin regulates proliferation and differentiation of osteoblastic cells

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Abstract

It has previously been reported that growth hormone secretagogues (GHS) may have a role in the regulation of bone metabolism in animals and humans. In this study we evaluated the effect of ghrelin, the endogenous ligand of GHS receptors, on the proliferation rate and on osteoblast activity in primary cultures of rat calvaria osteoblasts. In the same experiments, we compared the effects of ghrelin with those of hexarelin (HEXA) and EP-40737, two synthetic GHS with different characteristics. Both ghrelin and HEXA (10^{-11}-10^{-8} M) significantly stimulated osteoblast proliferation at low concentrations (10^{-10} M). Surprisingly, EP-40737 demonstrated an antiproliferative effect at 10^{-9}-10^{-8} M, whereas lower concentrations had no effect on cell proliferation. Ghrelin and HEXA significantly increased alkaline phosphatase (ALP) and osteocalcin (OC) production. At variance with these peptides, EP-40737 did not significantly stimulate ALP and OC. The mRNA for GHS-R1a receptors and the corresponding protein were detected in calvarial osteoblasts by RT-PCR and Western blot respectively, indicating that ghrelin and GHS may bind and activate this specific receptor. We conclude that endogenous ghrelin and synthetic GHS modulate proliferation and differentiation of rat osteoblasts, probably by acting on their specific receptor.

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Introduction

Growth hormone secretagogues (GHS) are synthetic compounds that elicit growth hormone (GH) release both *in vivo* and *in vitro* (Locatelli & Torsello 1997) through interaction with specific GHS receptors (GHS-R) (Petersenn 2002). Two GHS-R mRNA isoforms produced by alternative mRNA processing have been identified so far: the full-length 1a receptor and the truncated GHS-R1b type (Petersenn 2002). The GHS-R1a is the functional form of the receptor that mediates the neuroendocrine effects of GHS, whereas the functional significance, if any, of GHS-R1b remains to be elucidated. Recently, an endogenous ligand for GHS-R1a was cloned from rat and human stomach, and was named ghrelin. Ghrelin was shown to be a 28 amino acid peptide bearing the unique acylation on the Ser^3 residue (Kojima *et al.* 1999, 2001). Ghrelin, as well as many GHS, also participates in the regulation of energy homeostasis (Inui 2001), cardiovascular (Bodart *et al.* 1999, Locatelli *et al.* 1999) and gastric functions (Sibilia *et al.* 2002). However, the widespread expression of the genes encoding ghrelin and its cognate receptor in a variety of tissues including adrenals, prostate, thymus, hypothalamus, hippocampus and bone (Komatsu *et al.* 2000, Broglio *et al.* 2002, Guanapavan *et al.* 2002) strongly supports a potential for multiple biological activities of ghrelin (van der Lely *et al.* 2004). The possibility that ghrelin could be involved in the control of bone metabolism is also strengthened by the observation that gastrectomy induces osteopaenia (Bussabergher *et al.* 1938, Ivy 1940, Rümenappf *et al.* 1997) and that the acid-producing (oxyntic) mucosa (where ghrelin is synthesized) is important for bone metabolism (Lehto-Axtelius *et al.* 1998). Providing support for this hypothesis, we have previously reported that hexarelin (HEXA), a synthetic GHS, was capable of counteracting bone loss in male gonadectomized rats (Sibilia *et al.* 1999). Similarly, oral administration of GHS compounds was reported to increase bone turnover in humans (Svensson *et al.* 2001). A very interesting possibility is that the

protective effect of HEXA on bone could be due not solely to its GH–releasing activity but also to direct effects on bone cell activities.

In this study we tested whether the latter hypothesis is true by evaluating the effect of HEXA and EP–40737 (another GHS that stimulates GH secretion but not food intake in rats) on the proliferation rate and on osteoblast activity in primary cultures of rat calvaria cells. In the same experiments we compared the effect of HEXA and EP–40737 with those of ghrelin, the endogenous ligand of GHS-R1a. Furthermore, to assess the specificity of action of the compounds examined, the expression of GHS-R1a in our osteoblast cell line was determined by RT-PCR and Western blot.

Materials and Methods

Primary osteoblast cultures

All experimental protocols met the Italian Guidelines for the Use of Laboratory Animals which conform with the European Union Directive of November 1986 (86/609/EEC). The primary osteoblastic cells were obtained from rat calvariae explanted from E 21 fetuses as previously described by Bellows and coworkers (1987) with modifications. Briefly, calvaria were finely minced and incubated three times with 5 ml of an enzyme mixture containing 1 mg/ml collagenase type IV and 0·25% trypsin (1:250) (Sigma-Aldrich, Italy). Dispersed cells were collected after 20 min (population 1), 30 min (population 2) and 60 min (population 3) of incubation. An equivalent volume of fresh enzyme was added after each cell separation. Immediately after harvesting, an equal volume of Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) containing 10% of fetal bovine serum (FCS, Sigma-Aldrich) was added to the cell suspension to block the enzymatic activity. The cells were then pelleted by centrifugation, resuspended in DMEM and the three cell populations were pooled. The cells were then cultured in DMEM containing 10% FCS, 1% L-glutamine, 100 mg/ml penicillin and 10 IU streptomycin. The cells in culture were confirmed to be osteoblasts both by morphology and alkaline phosphatase (ALP) expression (see below). When confluence was reached, the medium was also supplemented with 50 µg/ml ascorbic acid and 3 mM β–glycerophosphate (Sigma-Aldrich) to trigger osteoblast differentiation.

The experiments were performed on cells at passage 6 when maximal ALP expression was found. The day before the experiment, the culture medium was removed and cells were maintained in DMEM without FCS for 24 h. Cells were then incubated with fresh DMEM containing HEXA (10^{-11}-10^{-9} M), ghrelin (10^{-11}-10^{-9} M), EP–40737 (10^{-11}-10^{-9} M), vitamin D (Vit D) (10^{-9} M) or FCS (10%) for 24–48 h.

[³H]Thymidine incorporation assay

The cells were plated at a density of $1·3 \times 10^4$ cells/cm² in 24-well plates. At confluence the cells were incubated for 24 h in DMEM without FCS. Fresh medium was then added containing different drugs and the cells were incubated for 24 h. [³H]Thymidine (1 µCi/well, specific activity 25 Ci/mmol, Amersham Pharmacia Biotech, UK) was added during the final 2 h of incubation (Ernst *et al.* 1989). After washing with DMEM, cells were incubated for 60 min with 5% cold trichloroacetic acid (Sigma-Aldrich) and harvested. Radioactivity was measured using a liquid scintillation counter (Wallac, Turku, Finland).

Biochemical determinations

Alkaline phosphatase The ALP activity was assessed by measuring the release of p-nitrophenol at 37 °C in 1 h when p-nitrophenylphosphate was used as a substrate (Sigma-Aldrich) (Farley & Jorch 1983). The enzymatic reaction was stopped with 50 mM NaOH and absorbance measured at A_{405} using an ELISA plate reader (SLT Lab Instruments, Hillsborough, NC, USA). The results were expressed as nmol/min/µg protein.

Osteocalcin production Quantitative determination of osteocalcin (OC) levels was performed after 48 h incubation using a rat osteocalcin EIA kit from Biomedical Technologies, Inc. (Stoughton, MA, USA). The inter- and intra–assay coefficients of variation were 7% and 4% respectively and the sensitivity of the assay was 0·5 ng/ml. Values were expressed as ng/mg protein.

Proteins The total protein content was measured on sonicated pelleted cells with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

Total RNA isolation and RT-PCR assay

Total RNA was extracted from primary osteoblastic cells (2×10^6 cells/plate) according to the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynsky & Sacchi 1987). The integrity of extracted RNA was ascertained by electrophoresis on agarose gel. Two hundred and fifty nanograms total RNA from each sample were subjected to reverse transcription with Moloney murine leukaemia virus reverse transcriptase (InVitrogen, Carlsbad, CA, USA) followed by amplification using specific primers based on the published sequence of rat GHS receptor (forward primer: AGC GCTACTTCGCCATC; reverse primer: CCGATGAG ACTGTAGAG). PCR analysis of total RNA yielded a DNA fragment of the expected length for the specific mRNA. To normalize results for differences in RNA sampling, an aliquot of the same RT reaction was used to amplify a rat glyceraldehyde-6-phosphate (GAPDH)

GHRELIN

HEXARELIN

EP-40737
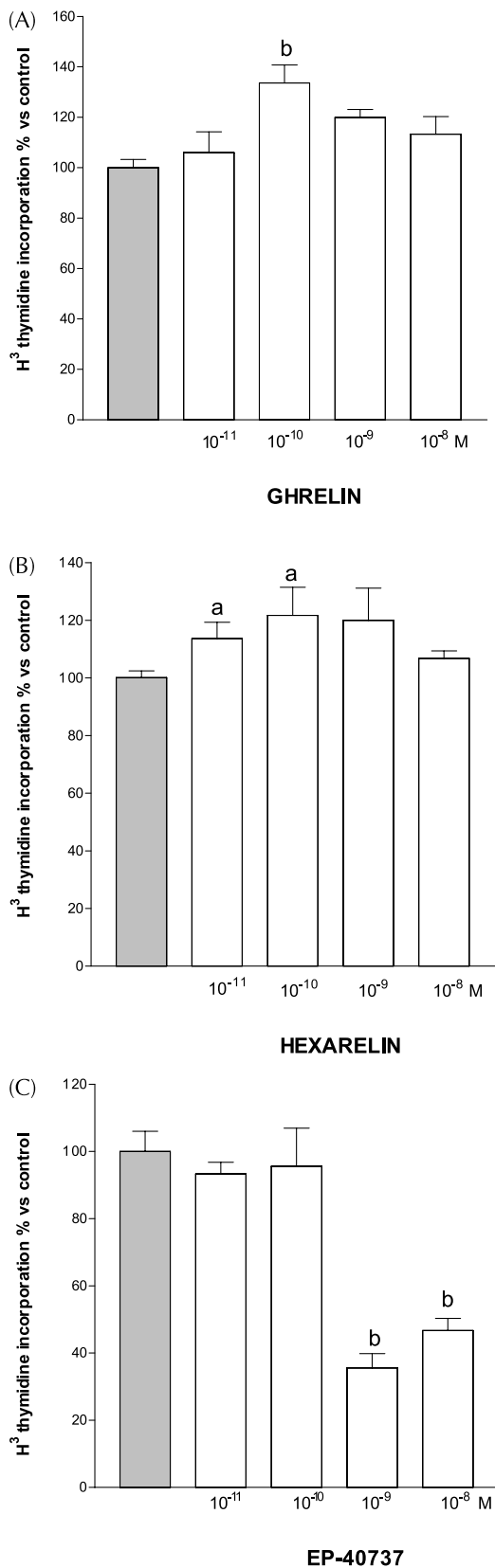
598-bp fragment. To ensure that PCR was performed in the linear amplification range, samples were initially analysed after 15, 17, 20, 25, 27, 30, 35, and 40 cycles (data not shown). For each factor, we chose the cycle number that gave half of the maximal amplification.

Western blotting

Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7·2, 0·1% SDS, 1% Nonidet P-40, 150 mM NaCl and 1% deoxycholic acid) containing protease inhibitor cocktail (Complete, Roche), homogenized by sonication (three 15-s bursts) and centrifuged at $18\,400 \times g$ for 5 min, after 20 min on ice. The protein concentration of the supernatants was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of each sample (30 μg or 60 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham). After blocking in 5% non-fat milk in phosphate-buffered saline (PBS) containing 0·2% Tween 20, blots were incubated with antibody anti-GHS-R (GHSR11-S, Alpha Diagnostic, S. Antonio, TX, USA) 1:1000, followed by reaction with secondary anti–rabbit horseradish peroxidase (HRP)-conjugate and ECL detection (Pierce Supersignal, Pierce).

Drugs

Hexarelin (HEXA), EP-40737 (D-Thr-D-Trp(2-Me)–Ala-Trp-D-Phe-Lys-NH$_2$) and ghrelin were a kind gift from Dr Romano Deghenghi (Europeptides, Argenteuil, France), and have been prepared by conventional solid–phase synthesis and purified to at least 98% purity by high-performance liquid chromatography by Neosystem (Strasbourg, France).

Statistical analysis

Statistical analysis was performed with a statistics package (PRISM, GraphPad Software, San Diego, CA, USA). All data are represented as the mean ± S.E.M. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test, with a probability of $P < 0·05$ considered statistically significant.

Results

Osteoblast proliferation

The viability of cells and the correct setting of culture conditions were tested by stimulating cell proliferation

Figure 1 Effect of different concentrations of ghrelin (A), hexarelin (B) and EP-40737 (C) on cultured rat osteoblasts proliferation. Data are expressed as a percentage of [^{3}H]thymidine incorporated into nonactively growing fetal rat osteoblasts at 24 h (controls, shaded bars). Means ± S.E.M. of 3–9 samples from the same cell population are reported. [a]$P < 0·05$, [b]$P < 0·01$ vs controls.
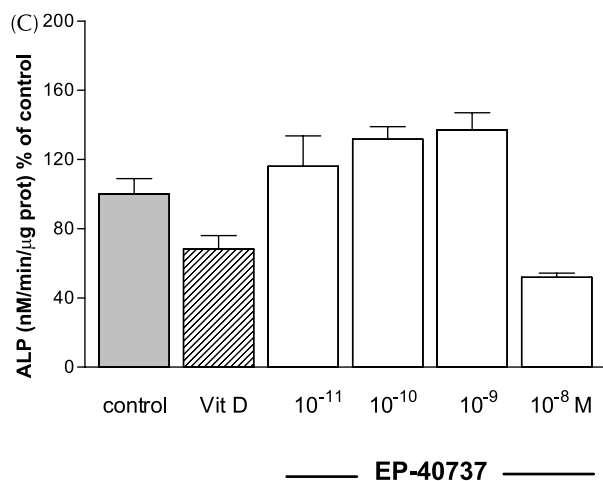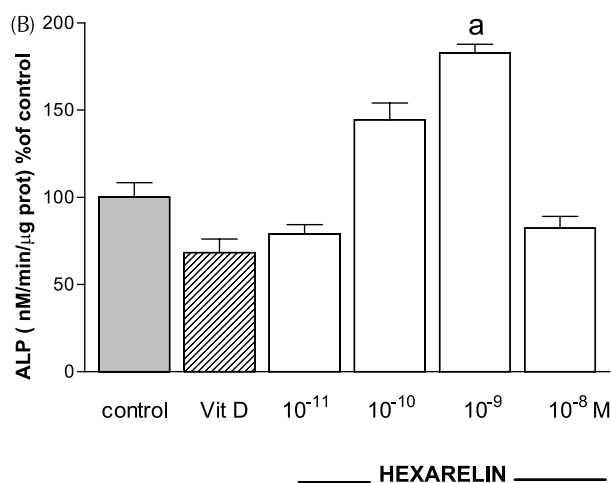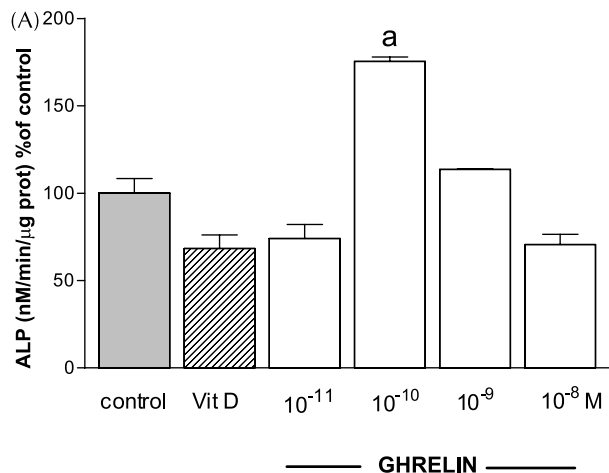
with DMEM containing 10% FCS. In these wells, [³H]thymidine incorporation was significantly increased up to 300% over that of controls (data not shown).

Figure 1 shows the effect of ghrelin, HEXA and EP-40737 on osteoblast proliferation. At a concentration of 10^{-10} M, both ghrelin and HEXA induced a significant stimulation of osteoblast proliferation, that reached an increase of 33% and 21% respectively over controls ($P<0.05$). The stimulation of proliferation did not increase further when the concentration of the peptides was elevated to 10^{-9} M (Fig. 1A,B).

Surprisingly, EP-40737 showed an antiproliferative effect in calvarial osteoblasts. In fact, when the cells were incubated with 10^{-8} and 10^{-9} M EP-40737 the incorporation of [³H]thymidine decreased significantly to approximately 40% of that measured in controls ($P<0.05$; Fig. 1C).

### Alkaline phosphatase activity and osteocalcin production

To ascertain whether ghrelin and synthetic GHS are capable of affecting osteoblastic cell differentiation, we measured the changes in ALP activity and OC production *in vitro*.

As shown in Fig. 2, ghrelin increased ALP activity in osteoblasts with a typical bell-shaped curve, and the maximal effect was reached when cells were incubated with 10^{-10} M ghrelin ($P<0.05$ vs controls, Fig. 2A). A similar curve was seen after addition of HEXA to cultured osteoblasts. In this case, however, the maximal stimulatory effect on ALP was reached at 10^{-9} M HEXA ($P<0.05$ vs controls, Fig. 2B). At variance with these peptides, the stimulation of ALP activity by EP-40737 was less marked and never reached statistical significance (Fig. 2C).

Responsiveness of the cells was confirmed by the addition of 10^{-9} M Vit D; in our setting this concentration significantly decreased ($P<0.05$) ALP specific activity to 65% of controls.

Figure 3 shows the results obtained by measuring OC production from cultured osteoblasts. As expected, Vit D at 10^{-9} M induced a significant increase of OC secretion. Both ghrelin and HEXA induced a consistent rise in OC secretion into the medium, although at different concentrations (Fig. 3A,B). A bell-shaped curve was obtained as a result of cell incubation with ghrelin, and the highest effect was obtained when the peptide was used at the concentration of 10^{-9} M. In contrast, HEXA was maximally effective at the lowest concentration tested (10^{-11} M).

Figure 2 Effect of different concentrations of ghrelin (A), hexarelin (B) and EP-40737 (C) on alkaline phosphatase (ALP) production in primary cultures of rat osteoblasts. Responsiveness of the cells was confirmed by the addition of 10^{-9} M Vit D. Data are expressed as a percentage of controls. Means+S.E.M. of 3–9 replicates are reported. [a]$P<0.05$ vs controls.
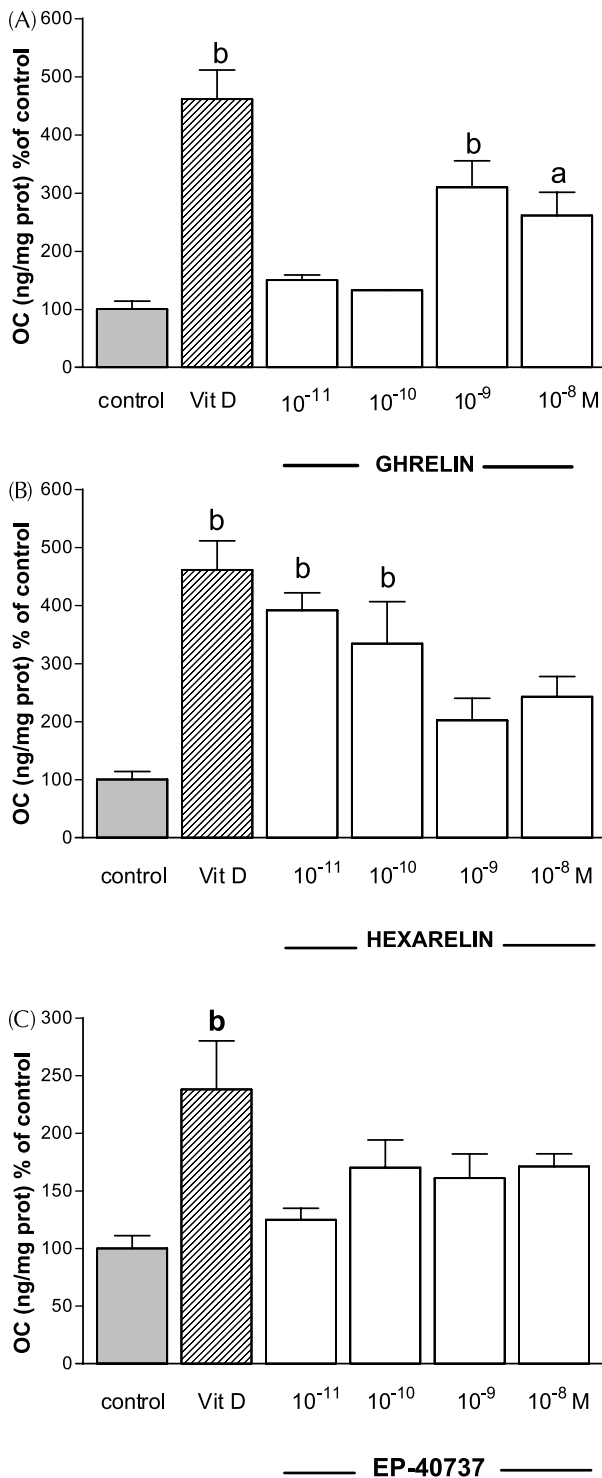
Figure 3 Effect of different concentrations of ghrelin (A), hexarelin (B) and EP-40737 (C) on osteocalcin (OC) production in primary cultures of rat osteoblasts. Vit D (10^{-9} M) was added as a control. Data are expressed as a percentage of controls. Means ± S.E.M. of 3–9 replicates are reported. [a]$P<0.05$, [b]$P<0.01$ vs controls.

Also in this instance, EP-40737 did not share the effects of the other GHS and did not significantly stimulate OC production from cultured osteoblasts (Fig. 3C).

GHS receptor expression in osteoblasts

The expression of specific mRNA for GHS receptor in fetal calvaria cells was investigated by using a standard RT-PCR. Amplification of GHS receptor mRNA gave rise to a sharp band of the expected length of 288 bp in calvaria cells harvested from passages 1 to 10 (Fig. 4). The effective translation of mRNA into the corresponding protein was demonstrated by Western blot from passages 5 to 10 (Fig. 5). In fact, a specific band of 44 kDa corresponding to the molecular weight demonstrated for the GHS receptor was present in all samples. Interestingly, at passage 10, a second band was strongly and specifically recognized by the anti-GHS receptor antibody, but its nature remains unknown (Fig. 5).

Discussion

Results of the present research are the first direct demonstration that rat calvarial osteoblasts express GHS-R1a receptors, considered the functional receptor for natural and synthetic GHS. The receptor has been detected by RT-PCR and by Western blot from the 1st to the 10th passage of osteoblasts. Furthermore, the present results demonstrate that ghrelin and HEXA stimulate osteoblast replication and increase ALP and OC production.

Previous reports have shown that both ghrelin and HEXA recognize with the same affinity the functional GHS-R1a receptor and that activation of this receptor stimulated an increase in $[Ca^{2+}]_i$ (intracellular calcium) and in IP_3 generation (Root & Root 2002). The effects of GHS on these second messengers correlate with their stimulatory effects on osteoblast proliferation since stimulation of the $PI/[Ca^{2+}]_i$ signal pathways are generally associated with increased DNA and collagen synthesis (Yamaguchi *et al.* 1988).

One intriguing finding is that ghrelin and HEXA stimulated osteoblast proliferation in a fashion that was not directly dose-dependent. In fact, the maximal stimulatory activity of ghrelin and HEXA was observed at a low concentration (10^{-10} M) but not at higher concentrations (10^{-8} and 10^{-9} M). It could be speculated that by increasing the dose, the peptides could recognize different receptor subtypes having inhibitory proliferative activity thus tempering the stimulatory action prevailing at low concentrations. Supporting this view are the results obtained with EP-40737, showing that the peptide exerts an inhibitory activity on cell proliferation when added at high concentrations. In this context it is worth noting that different binding affinities have been shown for ghrelin and HEXA on GHS-1a receptor (Guerlavais *et al.* 2003,)
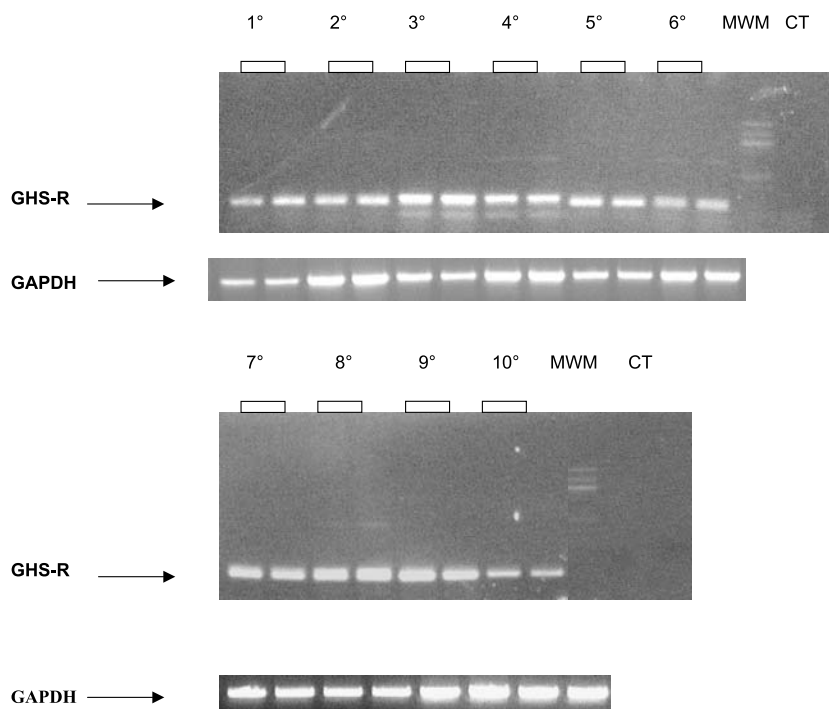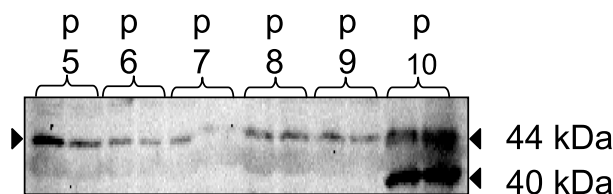
Figure 4 Expression of GHS-R1a mRNA in calvarial osteoblasts. Primary cultures of calvaria cells were harvested from passages 1 to 10. Total RNA extraction and RT-PCR were performed as described in Materials and Methods. Specific primers for GHS-R1a amplified a single band of the expected length of 288 bp in all samples (35 cycles). The lower panel shows amplification of GAPDH in aliquots of the same RT of the corresponding samples in the panel above. MWM, molecular weight marker; CT, control.

and that either stimulatory (Pettersson *et al.* 2002, Jeffery *et al.* 2003) or inhibitory (Ghè *et al.* 2002) effects on cell proliferation have been reported for these peptides.

The fact that EP-40737 behaves differently from these peptides could be related to the ability of the compound to recognize only the receptor subtype involved in anti-proliferative activity. Accordingly EP-40737 does not share all the biological activities of ghrelin and HEXA (Torsello *et al.* 1998, 2000).

In agreement with the different effects of the three GHS on osteoblast proliferation, EP-40737 does not modify the biochemical markers of osteoblast differentia-

Figure 5 Characterization of GHS receptor protein expression in calvaria cells. The cellular receptor expression was determined by Western blot as described in Materials and Methods. Calvaria cells were harvested from passages (p) 5 to 10. The 44 kDa band corresponds to the GHS receptor. A representative Western blot is shown.

tion, whereas ghrelin and HEXA significantly stimulate ALP activity and OC production. It would appear, therefore, that both the synthetic HEXA and the alleged natural ligand of the GHS receptors, ghrelin, modulate osteoblast differentiation. It has been reported that different signalling molecules are responsible for the regulation of osteoblast differentiation (Hakeda *et al.* 1987, Boguslawski *et al.* 2000) and it is known that ALP and OC production reflect different aspects of osteoblast differentiation and function. Thus it is likely that, by binding the GHS-R1a, ghrelin and HEXA could activate multiple signalling pathways that regulate, with different sensitivity, the osteoblastic functional activity. However, further investigations will be required to establish the mechanisms of GHS actions on osteoblast function and second messenger generation.

In line with this view, HEXA administered *in vivo* completely prevented the early decrease in serum ALP concentrations in gonadectomized rats, as a likely consequence of the direct activation of this enzyme in osteoblasts.

What would be the significance of these findings and what would happen in physiological conditions? The most likely agonist candidate for the GHS receptors on osteoblasts would be ghrelin. It has been known for a long

time that gastrectomy causes osteopaenia in experimental animals (Bussabergher *et al.* 1938, Ivy 1940, Rümenappf *et al.* 1997). Although a deficiency in calcium may contribute, it is unlikely that gastrectomy-evoked osteopaenia simply reflects calcium deficiency, since supplementation with $CaCl_2$ did not prevent the bone loss (Klinge *et al.* 1995, Rümenappf *et al.* 1997). The results support the view that the stomach is important for bone metabolism through mechanisms other than the regulation of intestinal calcium absorption. Interestingly enough, Lehto-Axtelius *et al.* (1998) demonstrated that the acid-producing (oxyntic) mucosa, which produces the most ghrelin, is the most important for bone metabolism. In view of these findings, we can suggest that ghrelin secreted from the stomach is conveyed through the circulation to its receptors located on bone.

An alternative hypothesis would be that the same bone cells are the source of ghrelin that would exert its effect through paracrine or autocrine mechanisms in a similar way as other local factors (cytokines, growth factors, prostaglandins, nitric oxide) (Canalis 1996). This view is supported by the single abstract report by Komatsu *et al.* (2000) who detected the ghrelin gene transcript in several different regions of mouse bone, including vertebrae, tibia, sternum and primary osteoblasts from calvaria.

At variance with our data, Sun *et al.* (2003) reported no role for ghrelin in the maintenance of bone density since ghrelin-null mice had normal bone mineral density and bone mineral content. Interestingly enough, food intake, body composition and reproductive function in these mice also did not differ from the wild-type littermates.

In view of the important regulatory role of ghrelin demonstrated for all these functions (van der Lely *et al.* 2004) it is likely that ghrelin-null mice would develop some compensatory mechanisms to overcome the lack of the peptide. However, it should be emphasised that evaluation of bone mineral density does not provide any information on osteoblast activity. In fact, the biochemical markers of osteoblast function, osteocalcin and ALP, were not determined in the ghrelin-null mice. Therefore, the possible physiological role of ghrelin in the control of osteoblast function cannot be ruled out and deserves further *in vivo* studies.

Acknowledgements

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