Preptin, another peptide product of the pancreatic β-cell, is osteogenic in vitro and in vivo


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The maintenance of the mechanical integrity of the skeleton depends on bone remodelling, the well-coordinated balance between bone formation by osteoblasts and bone resorption by osteoclasts. The coupled action of osteoblasts and osteoclasts is regulated by the action of many local and circulating hormones and factors. More recently, leptin-mediated central regulation of bone mass has been demonstrated (14). This appears to be mediated by a neurological rather than an endocrine mechanism (1). If there is uncoupling of the components of bone remodelling, such that bone resorption exceeds bone formation, bone loss occurs, leading to osteoporosis and fragility fractures. Current therapies for prevention of osteoporosis target osteoclast bone resorption in the main, but these agents have limited ability to improve bone mass. There is great interest, therefore, in agents that may positively affect bone mass by stimulating bone formation.

Recently, the importance of nutritional hormones in maintaining skeletal health has been recognized. This is reflected in the lower prevalence of osteoporosis in those with obesity. Thus hormones circulating at higher levels in obesity have received attention as potential anabolic agents in bone, including the products of pancreatic β-cells and of adipocytes. Obesity is associated with hyperinsulinaemia, arising from resistance to the hypoglycaemic effects of insulin. At least two other peptides, amylin and preptin, are cosecreted with insulin from the β-cell (4, 5) and would be expected to circulate in increased concentrations in obesity. Insulin and amylin are already known to have effects on bone cells in vitro and in vivo (6, 7, 8, 11). Both are anabolic to osteoblasts, and amylin, which belongs to the calcitonin family, also inhibits osteoclast bone resorption. Preptin is a recently discovered 34-amino acid peptide corresponding to Asp69-Leu102 of pro-IGF-II. Preptin is a recently discovered 34-amino acid peptide complexed with IGF-binding protein-2, which belongs to the calcitonin family, also inhibits osteoclastic bone resorption. Preptin is a recently discovered 34-amino acid peptide corresponding to Asp69-Leu102 of pro-IGF-II peptide (4). It appears to act as a physiological amplifier of glucose-mediated insulin secretion, but its effects on the skeleton are unknown. The present study investigates the effects of preptin on osteoblasts and osteoclasts in organ culture and in an in vivo local injection model. We demonstrate that preptin is anabolic to bone in vitro and in vivo but, unlike amylin, does not regulate osteoclast activity.

Materials and Methods

Materials. Rat and human preptin were synthesized by Auspep Pty, Parkville, Victoria, Australia, using standard chemical methods. Osteoblast-like cell cultures. Primary cultures of osteoblasts were used to assess the effects of preptin on osteoblast-like cells. Primary rat osteoblast-like cells were derived from sequential collagenase digestion of 20-day fetal rat calvariae, as described earlier (10). Cells of the human osteoblast-like cell line (SaOS-2), murine osteoblast-like cell line (MC3T3-E1), and murine fibroblast cell line (3T3-Swiss) were grown in a similar manner. Cell proliferation was monitored in growth-arrested cells by measurements of thymidine incorporation and cell number. In experiments designed to assess the effect of pharmacological inhibitors of intracellular signaling on the proliferative actions of preptin, inhibitor-only controls were included, and the proliferative response to preptin was assessed in both the absence and the presence of the inhibitor. Osteoblast apoptosis was assessed using the TUNEL method (using the kit DeadEnd; Promega, Madison, WI) on serum-deprived cells (13). These osteoblast cultures were also prolonged, permitting observation of osteoblast differentiation through the formation of bone nodules. These cultures required the presence of 50 μg/ml ascorbic acid 2-phosphate and 10 mM sodium β-glycerophosphate. Medium was changed every third day. Preptin (and DNA synthesis) of primary fetal rat osteoblasts and osteoblast-like bone. Preptin dose-dependently stimulated the proliferation (cell number and DNA synthesis) of primary fetal rat osteoblasts and osteoblast-like cell lines at periphysiological concentrations (>10−11 M). In addition, thymidine incorporation was stimulated in murine neonatal calvarial organ culture, likely reflecting the proliferation of cells from the osteoblast lineage. Preptin did not affect bone resorption in this model. Preptin induced phosphorylation of p42/p44 MAP kinases in osteoblastic cells in a dose-dependent manner (10−8–10−10 M), and its proliferative effects on primary osteoblasts were blocked by MAP kinase kinase inhibitors. Preptin also reduced osteoblast apoptosis induced by serum deprivation, reducing the number of apoptotic cells by >20%. In vivo administration of preptin increased bone area and mineralizing surface in adult mice. These data demonstrate that preptin, which is cosecreted from the pancreatic β-cell with amylin and insulin, is anabolic to bone and may contribute to the preservation of bone mass observed in hyperinsulinaemic states such as obesity.
Immunoblotting. Immunoblotting was performed as previously described (16). In experiments designed to determine the effect of inhibitors of signal transduction, the cells were pretreated with the inhibitor for 30 min prior to addition of preptin. Quantification of p42/44 MAPK phosphorylation, normalized for total p42/44 MAPK protein, was undertaken using laser scanning densitometry (Fujifilm LAS-3000).

Osteoclast cell cultures. Isolated mature osteoclasts cultured on devitalized bone chips were isolated from long bones of 1-day-old rats (9). The amount of bone resorbed was measured by reflective microscopy and image analysis. The results for each bone slice were expressed as the ratio of the number of pits to the number of osteoclasts. There were 6–12 bone slices in each group, and each experiment was repeated two or three times.

Bone marrow cultures. Bone marrow cultures supplemented with 1,25(OH)2D3 to the tissue culture plates were fixed with citrate-acetone-formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using Sigma Kit no. 387-A. TRAP-positive multinucleated cells were counted in all wells. There were at least eight wells for each group and each experiment was repeated three or four times.

Bone organ culture. Mice were injected subcutaneously with 5 μCi 45Ca (Amersham Biosciences UK, Buckinghamshire, UK) at 2 days of age, and hemicalvariae were dissected out 4 days later. Hemicalvariae were preincubated for 24 h in medium 199 (GIBCO, Invitro-
**RESULTS**

Preptin stimulates the proliferation and differentiation of osteoblasts. Preptin, similar to other products of the pancreatic β-cell, insulin and amylin, stimulated osteoblast proliferation. At concentrations of $>10^{-11}$ M, rat and human preptin increased thymidine incorporation and cell counts in primary cultures of rat osteoblast-like cells at 24 h (Fig. 1, A and B). The same range of concentrations of preptin stimulated proliferation of: MC3T3-E1 cells (Fig. 1C) and SaOS-2 cells (Fig. 1D).

The aforementioned studies of preptin action on osteoblast proliferation were complemented by an assessment of its action on differentiation of these cells. These studies were performed using 3-wk cultures of primary rat osteoblasts to assess bone nodule formation, a process that involves bone matrix deposition and mineralization, both of which are functions of differentiated osteoblasts. Preptin dose-dependently increased the number of nodules formed (Fig. 2).

In addition, thymidine incorporation was stimulated in murine neonatal calvarial organ culture, likely reflecting the proliferation of cells from the osteoblast lineage (Fig. 3).

**Fig. 3.** Effects of r-preptin on thymidine incorporation in neonatal mouse calvariae organ culture. Thymidine incorporation, reflecting osteoblast proliferation, was increased, but there was no effect on bone resorption in this model. Data are means ± SE from a representative experiment. *Significantly different from control ($P < 0.05$).

Preptin does not affect osteoclast activity. Preptin did not affect bone resorption in the murine calvarial organ culture model; neither did it regulate activity of isolated mature osteoclasts (data not shown). Similarly, preptin did not affect osteoclast development in a murine bone marrow culture assay, as there were no significant differences from control in the number of TRAP-positive multinucleated cells formed over the 7-day culture period, in the presence of preptin from concentrations of $10^{-11}$ to $10^{-7}$ M.

Preptin osteoblast mitogenic activity is inhibited by pertussis toxin but not by amylin receptor blocker. The receptor for preptin has not been identified. Amylin-(8–37) blocks the mitogenic activity of amylin, insulin, and IGF-I (12); however, this blocker did not affect the proliferative response to preptin (Fig. 4A), nor did an IGF-I neutralizing antibody to the receptor

**Fig. 4.** A: preptin-stimulated osteoblast proliferation is not inhibited by blockade of the amylin receptor, using the amylin receptor blocker amylin-(8–37), as judged by cell number (left) and thymidine incorporation (right). B: preptin-stimulated osteoblast proliferation is inhibited by the G inhibitor pertussis toxin (PTx). Data are means ± SE. *Significantly different from control ($P < 0.01$).
However, the receptor mediating the proliferative actions of preptin is likely to be G protein-linked receptor coupled to G\textsubscript{i} proteins, since pertussis toxin inhibits the mitogenic actions of preptin in osteoblasts (Fig. 4B).

**Preptin-induced osteoblast mitogenesis involves activation of p42/44 MAP kinases.** We examined whether p42/44 MAP kinases were involved in preptin-induced mitogenic signaling in osteoblasts. As shown in Fig. 5, A and B, preptin induced phosphorylation of p42/44 MAP kinases in osteoblastic cells in a time- and dose-dependent (10\textsuperscript{-8}-10\textsuperscript{-10} M) manner. Pretreatment of primary rat osteoblastic cells with either PD-98059 or U-0126 inhibited preptin-induced osteoblast mitogenesis (Fig. 5C). PD-98059 and U-0126 are structurally unrelated inhibitors of MEK, the kinase that specifically phosphorylates and activates the p42/44 MAP kinases.

**Preptin promotes osteoblast survival.** We also assessed preptin’s effects on apoptosis of primary rat osteoblasts induced by serum deprivation. Apoptotic cells were detected by light microscopy using a modified TUNEL assay. Preptin had antiapoptotic effects at 10\textsuperscript{-8} M with a treatment-to-control ratio of 0.78 ± 0.08 (P < 0.05; Fig. 6).

**Preptin increases bone growth in vivo.** The anabolic effect of preptin on osteoblasts in vitro suggested that it might have positive effects on bone mass in vivo. To address this question, we administered preptin or vehicle over the right hemicalvaria of adult male mice for 5 consecutive days. These data are shown in Fig. 7. Preptin increased the extent of the mineralizing surface (Fig. 7A) and double-labeled surface (Fig. 7B) in a dose-dependent fashion (assessed using the 1st and 3rd fluorescent labels). Bone area was also dose-dependently increased.

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**Fig. 5.** Preptin signals osteoblast mitogenesis via activation of p42/44 MAPKs. A: lysates of primary rat osteoblastic cells treated with 1 nM preptin for indicated times (lanes 1–5) or for 10 min with the indicated concentration of preptin (lanes 7–11) were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (top) and an antibody to total p42/44 MAPKs (bottom). B: p42/44 MAPK phosphorylation in lysates of primary rat osteoblastic cells treated for 10 min with indicated doses of preptin was quantified by laser scanning densitometry and normalized to total p42/44 MAPK protein. Pooled data from 3 separate experiments are presented as fold change over control values. Data are means ± SE. *P < 0.05 vs. control; **P < 0.01 vs. control. C: osteoblast mitogenesis ([\textsuperscript{3}H]thymidine incorporation) is inhibited by MAPK kinase inhibitors PD-98059 and U-0126. Pooled data from 3 separate experiments are presented as fold stimulation of [\textsuperscript{3}H]thymidine incorporation over control values. Data are means ± SE. ns, Not significant.
DISCUSSION

This study demonstrates that preptin, a 34-amino acid peptide hormone secreted from the pancreatic β-cell, is anabolic to bone. In vitro, preptin acts on osteoblast-like cells to stimulate proliferation, differentiation, and survival. However, preptin does not affect either osteoclastogenesis or mature osteoclast activity. Collectively, the effects of preptin in vitro suggest that it might be anabolic to bone in vivo, a suggestion we confirmed in an adult murine hemicalvarial local injection model. Preptin increased calvarial bone area after only five injections, as well as increasing double-labeled and mineralizing surfaces.

The current results suggest that preptin may have both physiological and pathophysiological relevance to skeletal metabolism. A considerable body of evidence suggests that increased body weight and/or fat mass is associated with higher bone density and reduced risk of fragility fracture (7, 18, 19). Recently, several hormones that regulate or reflect nutritional status, such as insulin (20), leptin (19), amylin (7), and others (3, 25), have been found to impact directly on bone metabolism. In general, the accumulated evidence from these studies is consistent with the hypothesis that the increased levels of several nutrition-related hormones, derived from the pancreatic β-cell and adipocyte, that are observed in the peripheral circulation of overweight subjects contribute to the higher bone mass and lower fracture risk associated with obesity. Preptin was recently purified from secretory granules isolated from cultured β-cells, along with insulin and amylin. All three hormones are involved in glucose metabolism, and preptin may be a physiological amplifier of glucose-mediated insulin secretion (4). Insulin and amylin are anabolic to osteoblasts, and amylin also inhibits osteoclastic bone resorption. Preptin joins these hormones as a bone-active factor.

Preptin corresponds to Asp^69-Leu^102 of the 156-amino acid precursor of IGF-II, known as IGF-IIE. The circulating mature form of IGF-II is amino acids 1–67 of the prohormone (2, 4, 22). IGF-II has potent anabolic effects on bone, and IGF-IIE has a similar profile of biological activities (15, 23). An 18-kDa fragment of IGF-IIE, which includes amino acids 89–101, has been implicated in the osteosclerosis observed in patients with chronic hepatitis C infection, an acquired condition in which biochemical and histological indexes of bone formation are elevated, and bone mineral densities are elevated as much as twofold (24). However, increased bone mass is not seen in patients with tumors that produce a shorter form of IGF-IIE with immunoreactivity for amino acids 78–88 but not for 89–101 (17). These observations provide evidence that high levels of a peptide contained within the sequence of intact preptin may dramatically increase bone density in vivo. This suggests that the effects of preptin on bone cells that we observed in the present study may be associated with anabolic effects on bone mass in normal human physiology. The nature of the preptin receptor remains unknown; however, our data by preptin, such that the 16.5-μg dose induced significant changes from those observed in control animals (Fig. 7C).

Fig. 6. Effect of preptin on apoptosis observed in response to a 24-h period of serum deprivation in cultures of primary rat osteoblast-like cells, as judged by the number of TUNEL-positive cells. Data are means ± SE. *Significantly different from control (P < 0.05).

Fig. 7. Effects of preptin administered locally over the calvaria of adult male mice daily for 5 days in the doses indicated. Animals were killed 10 days later. A: MS/BS, the fraction of total bone surface at which mineralization is taking place. B: dLS/BS, the fraction of the total bone surface that has double fluorochrome labels. C: BAr, total bone area. Data are means ± SE. *Significantly different from control (P < 0.05).
suggest that preptin does not act through one of the putative amylin receptors, nor does its proliferative effect involve activation of the IGF-I receptor, as we observed with amylin and adrenomedullin (12). Our data suggest that preptin signals osteoblast proliferation through a G protein-coupled receptor that activates G_i-dependent phosphorylation of p42/44 MAP kinases. Activation of p42/44 MAP kinases (ERKs) is a common feature of proliferative signals initiated by a variety of extracellular agents (21). In this study, we have demonstrated that p42/44 MAP kinases are involved in preptin-induced mitogenic signaling in osteoblasts, since inhibitors of the p42/44 MAP kinase pathway abrogated the mitogenic effect.

In summary, preptin is a novel bone-active peptide hormone secreted from the pancreatic β-cell that may act in concert with the other β-cell hormones insulin and amylin to stimulate bone formation in hyperinsulinemic states such as obesity. As preptin contains within it the peptide sequence of the IGF-II fragment that likely underpins the high bone mass of some patients with chronic hepatitis C, our results also provide additional evidence for anabolic skeletal effects of peptides derived from pro-IGF-II.

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GRANTS

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REFERENCES