Amylin inhibits ovariectomy-induced bone loss in rats

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Abstract

Amylin (AMY), a peptide co-secreted with insulin by pancreatic β-cells, inhibits bone resorption and stimulates osteoblastic activity. The ovariectomized (OVX) rat is an established animal model for human osteoporosis. Thus, the present experiment was performed to study the effects of AMY on estrogen deficiency-induced bone loss in rats. Thirty-one 6-month-old Wistar rats were randomized by body weight (BW) into two groups. The first underwent surgical OVX ($n=21$). The second was sham-operated (SH; $n=10$). Sixty days after surgery, 11 OVX rats were s.c. injected with rat AMY (3 μg/100 g BW/day, for 30 days; OVX+AMY), and 10 with solvent alone in the same way (0·15 ml/100 g BW; OVX). Each rat, housed in an individual cage, was fed daily the mean quantity of diet consumed the day before by SH rats. This diet contained 0·24% calcium and 0·16% phosphorus. The 31 animals were killed on day 90. No difference in daily weight gain and BW was observed between groups. Neither AMY treatment nor OVX had any significant effect upon femoral morphology, femoral failure load, diaphyseal femoral density (representative of cortical bone) and total femoral calcium content. Nevertheless, both distal metaphyseal (representative of cancellous bone) and total femoral bone densities were higher in SH and OVX+AMY than in OVX rats. The highest plasma osteocalcin concentration was measured in OVX+AMY rats. Simultaneously, urinary deoxypyridinoline excretion was lower in OVX+AMY than in OVX rats. These results indicate that in OVX rats, AMY treatment inhibited trabecular bone loss both by inhibiting resorption and by stimulating osteoblastic activity.

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Introduction

Amylin (AMY) is a 37 amino acid hormone structurally similar to calcitonin-gene-related peptide (CGRP) (Cooper *et al.* 1987) and to the calcitonins at the N- and C-termini (Young *et al.* 1995). AMY is mostly expressed in pancreatic β-cells, from which it is co-secreted with insulin in response to nutrient stimuli (Ogawa *et al.* 1990). AMY is absolutely or relatively deficient in conditions of β-cells loss, such as type 1 diabetes and late type 2 diabetes mellitus. AMY receptors of the type found in nucleus accumbens (Beaumont *et al.* 1993) and area postrema have been elusive. Their pharmacology has recently been shown to be produced by the co-expression of calcitonin C1a receptors and receptor activity modulating protein (RAMP1) (Muff *et al.* 1995, Christopoulos *et al.* 1999) in a manner similar to the formation of CGRP receptor from calcitonin receptor-related receptor and RAMP1 (MacLatchie *et al.* 1998).

Despite nearly 60 reported actions of AMY, its physiological role is poorly understood. Its most potent (physiological) metabolic actions, including inhibition of gastric emptying, inhibition of food intake, inhibition of pancreatic enzyme secretion and inhibition of glucagon secretion, are consistent with a primary role in controlling the rate of glucose appearance in the plasma (Young 1997). In this way, AMY's function appears to complement that of insulin, which primarily controls glucose disposal.

In addition, AMY has potent effects to lower plasma calcium (Ca) concentration and inhibit osteoclast activity (Datta *et al.* 1989, MacIntyre 1989), and is reported to stimulate osteoblast growth (Cornish *et al.* 1995, 1998*b*, Romero *et al.* 1995). Although AMY is less potent than calcitonin at calcitonin receptors, it circulates at higher concentrations, and could represent a significant signal at such receptors (Zaïdi *et al.* 1993). Some of the AMY's skeletal actions appear to be mediated via mechanisms distinct from those of calcitonin (Alam *et al.* 1993). The lack of AMY in type 1 diabetes mellitus has been proposed as a mechanism explaining susceptibility to osteopenia in those patients (Zaïdi *et al.* 1993) and treatment of type 1 diabetic subjects for 1 year with the AMY agonist pramlintide resulted in improvement of biochemical bone markers (Bone *et al.* 1999), particularly in postmenopausal female subjects. The reason that individuals with type 2 diabetes are not generally osteopenic is also

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probably substantially contributed to by the fact that their body weight (BW) tends to be high, in contrast to patients with type 1 diabetes. Moreover, patients with type 2 diabetes typically display variable (but non-zero) fasting plasma AMY concentrations that are not modulated by meals (Van Daele *et al.* 1995).

The aim of the present study was to investigate the effects of AMY in a commonly used animal model of post-menopausal osteoporosis, the ovariectomized (OVX) rat (Kalu 1991, Wronski & Yen 1991). Because OVX can increase food intake and body mass, and thereby affect bone density, while AMY can reduce food intake, the experiments were controlled by pair-feeding. They show effects of AMY on bone that indicate both inhibition of resorption and stimulation of osteoblasts.

Materials and Methods

Animals and treatments

These experiments were carried out in accordance with current legislation on animal experiments in France. The OVX rat develops osteopenia rapidly, within 3 months after surgery (Kalu 1991, Wronski & Yen 1991). Thus, 31 virgin female Wistar rats weighing 324 ± 4 g (mean ± S.E.M.) were used at 6 months of age. On day 0 of the experiment, they were randomized by BW into two groups. Then, under chloral anesthesia, the first group underwent surgical OVX ($n=21$). Other animals were sham-operated (SH, $n=10$). Sixty days after surgery, among the 21 OVX rats 11 were s.c. injected with synthetic rat AMY (3 μg/100 g BW per day, for 30 days; OVX+AMY) and 10 with solvent alone (0·9% (w/v) NaCl containing 0·01% BSA; OVX). Each rat was housed individually in a plastic cage allowing separate collection of urine and feces, at 21 °C, with a 12 h light:12 h darkness cycle. Low-Ca feeding has been accepted as a method of increasing bone resorption and as one of the experimental models to reduce bone mass (Salomon 1971, Sissons *et al.* 1984). Thus, to stimulate bone resorption, the animals were fed a semi-purified Ca-deficient diet containing corn starch (64%), purified casein (18%), alphacel non-nutritive bulk (10%), ground nut oil (2·5%), rape oil (2·5%) and vitamins and mineral mixture (3%) (Ca, 0·24%; phosphorus (P), 0·16%). To prevent hyperphagia induced by OVX, the individual daily consumption was measured and each rat received the mean quantity of the chow consumed by SH rats during the previous day (about 20 g/rat per day). Each animal was weighed weekly. Urine was collected during a 24 h period on day 89 of the experimental period to measure the excretion of Ca, P and deoxypyridinoline (DPD), a marker for bone resorption (Robins 1994).

Rats were killed by cervical dislocation on day 90. Blood was collected by cardiac puncture. After centrifugation, plasma was harvested and frozen until analysis. The success of OVX was confirmed by a marked atrophy of uterine horns in both OVX and OVX+AMY rats. Femurs were cleaned from adjacent tissue and used for physical and chemical measurements.

Physical measurements

Femoral mechanical testing Immediately after collection, the length of the right femur and the mean diameter of the femoral diaphysis were measured using a caliper. Due to the irregular shape of the femoral diaphysis, the femoral diameter used in the calculation was the mean of the greatest and the smallest femoral diaphysis diameters. Then, each bone was placed in 0·9% NaCl at 4 °C. The mechanical resistance of the femoral bones was determined 24 h later, by using a three-point bending test. Each bone was secured on the two lower supports (diameter 4 mm, length 20 mm) of the anvil of a Universal Testing Machine (Instron 4501, Instron, Canton, MA, USA). The upper roller diameter was 6 mm. The cross-head speed for all the tests was 0·5 mm/min. The load at rupture was automatically determined by the Instron 4501 software. To ensure comparable testing sites, the femur was always mounted so that the cross-head was applied just in the middle of the bone. Using female rats, the span of the specimen that was loaded was 20 mm to guarantee that 85–90% of the flexure of the bone was due to bending. This method of testing has been previously validated by using Plexiglas standard probes (Turner & Burr 1993). Results are expressed in newtons.

Bone mineral density Dual energy X-ray absorptiometry measurements were made with a Hologic QDR-4500A X-ray bone densitometer (Hologic France, Massy, France). Total femoral bone mineral density (T-BMD) was determined. Furthermore the BMDs of two subregions, one corresponding to the distal metaphyseal zone (M-BMD), which is rich in cancellous bone, and the other to the diaphyseal zone (D-BMD), which is rich in cortical bone, were measured (Pastoureau *et al.* 1995).

Biochemical analysis

Marker of osteoblastic activity Plasma osteocalcin (OC) concentrations were measured by homologous RIA using rat OC standard, goat anti-rat OC antibody, ^{125}I-labelled rat OC and donkey anti-goat second antibody (Biochemical Technologies kit, Stoughton, MA, USA). The lowest limit of detection was 60 pg/ml, and the intra- and inter-assay variations were 6·5 and 8·2% respectively.

Marker of bone resorption DPD in urine collected during the 24 h before killing was measured by an IRMA assay using a Pyrilinks-D kit (Metra Biosystems, Mountain View, CA, USA). The assay of DPD required the addition of 50 μl of urine sample (or DPD standard or control) to

Table 1 Femoral length and mean femoral diameter in SH, OVX and OVX+AMY rats (means ± S.E.M., no significant difference was observed between groups)

	OVX+AMY	OVX	SH
Length (mm)	37·3 ± 0·4	36·9 ± 0·4	36·5 ± 0·6
Diameter (mm)	3·80 ± 0·07	3·80 ± 0·05	3·87 ± 0·05

Table 2 Plasma Ca concentration, urinary Ca excretion and femoral Ca content in SH, OVX and OVX+AMY rats (means ± S.E.M.)

	OVX+AMY	OVX	SH
Plasma Ca (mM)	3·85 ± 0·04*	4·04 ± 0·03	3·94 ± 0·05*
Urinary Ca (mg/day)	3·3 ± 0·3	3·3 ± 0·5	4·2 ± 0·2
Femoral Ca (mg)	149·4 ± 4·3	150·3 ± 4·3	158·4 ± 7·9

*P<0·05 vs OVX.

each well of a DPD-coated microplate. The monoclonal antibody against DPD was added to the plate, and the free DPD in urine competed with the DPD coated on the plate for the antibody. A second antibody conjugated to alkaline phosphatase (goat anti-rabbit immunoglobulin G alkaline phosphatase) was added to bind with antibody against DPD. A substrate, p-nitrophenylphosphate, was added to produce a yellow color. Optical density was measured at 405 nm. In our experimental conditions, the lowest limit of detection for the assay was 3 nmol. The intra- and inter-assay variations were 6 and 8% respectively. Results are expressed as nanomoles DPD per millimole creatinine (Robins 1994). The urinary creatinine assay was used to adjust DPD values for variation in urine volume. This assay was based on a modified Jaffé method in which picric acid forms a colored solution in the presence of creatinine (Cook 1975).

Ca Ca was determined by atomic absorption spectrophotometry (Perkin Elmer 401, Norwalk, CT, USA) in plasma, urine and ashed bone samples diluted with 0·1% lanthanum chloride solution. P was measured by colorimetry.

Statistics

Results are presented as means ± S.E.M. A parametric one-way ANOVA was used to test for any differences among the groups. If the result was found significant (P<0·05), the Student–Newman–Keuls multiple comparison test was then used to determine the specific differences between group means. If a parametric ANOVA was not feasible (when there were significant differences between the standard deviations of the groups, tested by Kolmogorov–Smirnov test), a Kruskall–Wallis test followed by the Mann–Whitney Wilcoxon U-test was used to compare differences between groups.

Results

During the 90 days of the experimental period the mean BW of the 31 rats increased from 323 ± 10 g to 379 ± 11 g (P<0·05). No significant difference in daily weight gain or BW was observed between groups.

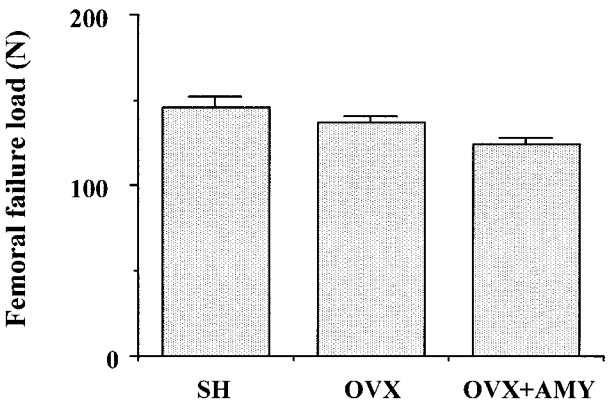

Figure 1 Femoral failure load measured in SH, OVX and OVX+AMY rats. Means ± S.E.M., no significant difference was observed between groups.

At necropsy, no significant difference in femoral length and mean femoral diameter was observed between groups (Table 1). In the same way, femoral failure load was not different in the three groups of rats (Fig. 1).

However, T-BMD was lower in OVX than in both SH and OVX+AMY rats. No significant difference in D-BMD was observed between groups. M-BMD was lower in OVX than in SH or OVX+AMY rats. M-BMD was slightly but significantly lower in OVX+AMY than in SH rats (Fig. 2). No significant difference in femoral Ca content or urinary Ca excretion was observed between groups. Plasma Ca concentration was higher in OVX than in SH or OVX+AMY rats, but it was not different in SH or OVX+AMY rats (Table 2).

The highest value for plasma OC concentration (22·3 ± 0·8 ng/ml) was measured in OVX+AMY rats. It was not different from that simultaneously measured in OVX rats, but it was higher than in SH rats. Urinary DPD excretion measured in OVX (166 ± 12 nmol DPD/mmol creatinine) was higher than that measured in OVX+AMY or SH rats. It was also slightly but significantly higher in OVX+AMY than in SH (Fig. 3).

Discussion

In the present study, treatment of OVX rats with 3 μg/100 g BW per day of rat AMY for 30 days fully or partly

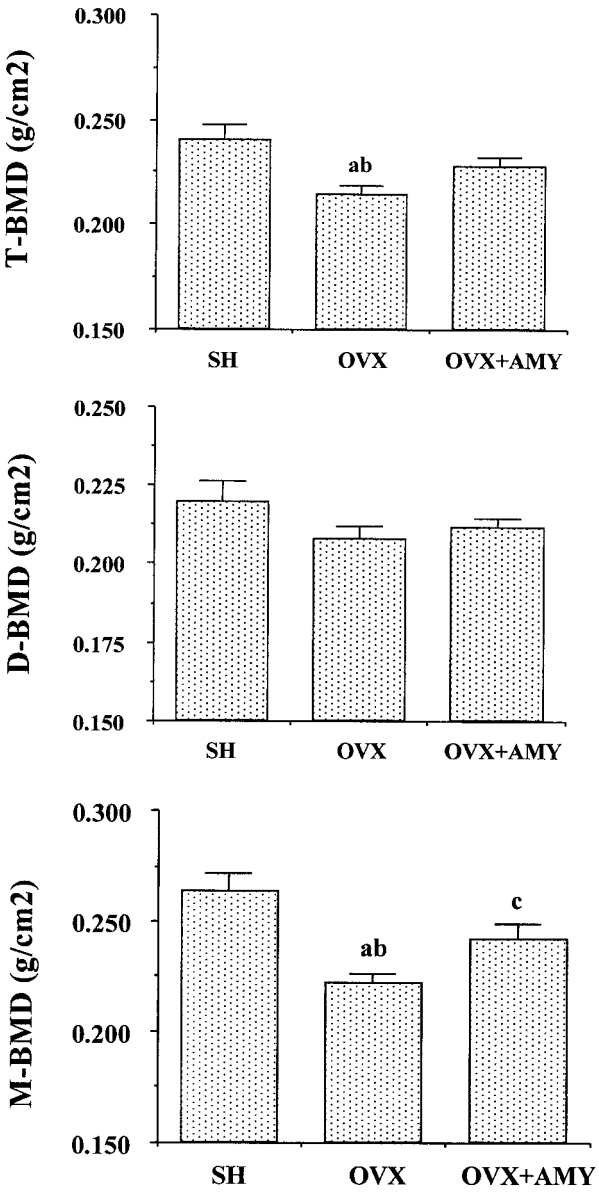

Figure 2 T-BMD, D-BMD and M-BMD femoral density measured in SH, OVX and OVX+AMY rats. Means ± S.E.M., $^{a}P<0{\cdot}01$ vs SH; $^{b}P<0{\cdot}05$ vs OVX+AMY; $^{c}P<0{\cdot}05$ vs SH.

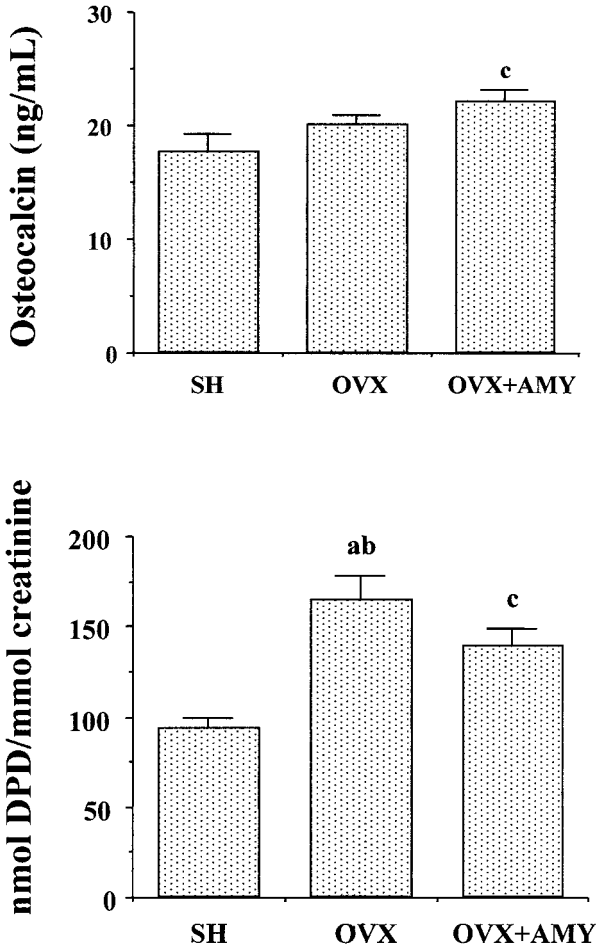

Figure 3 Plasma OC concentration and urinary DPD excretion measured in SH, OVX and OVX+AMY rats. Means ± S.E.M., $^{a}P<0{\cdot}01$ vs SH; $^{b}P<0{\cdot}05$ vs OVX+AMY; $^{c}P<0{\cdot}05$ vs SH.

restored towards normal (as exemplified in SH rats) several physical and biochemical marker abnormalities that had developed during the 90 days after OVX. Plasma Ca concentration, elevated in OVX rats, was restored to the levels in SH rats with AMY (Table 2). Total and metaphyseal femoral density, but not diaphyseal density, was partly restored with AMY treatment (Fig. 2). Urinary DPD excretion was partly normalized by AMY treatment, indicating a reduction in resorptive activity, and plasma OC was elevated in AMY-treated animals, consistent with a stimulation of bone formation (Fig. 3). There were no significant differences between groups in femoral failure load, femoral Ca content and urinary Ca excretion (Fig. 1; Table 2).

While the 3 μg/100 g s.c. injection employed in the present experiment is predicted from kinetic studies (Young *et al.* 1996) to have raised plasma AMY concentrations to a supraphysiological 1 nM, the mean increment in 24 h exposure to circulating AMY can be calculated to be approximately 35 pM, a 2- to 3-fold increase over physiological concentrations. Thus, the present studies do not preclude a physiological, or near-physiological, effect of AMY on bone.

In vivo, AMY causes hypocalcemia in rats and rabbits and, *in vitro*, it inhibits bone-resorbing activity of rat osteoclasts (Datta *et al.* 1989). In the same way, in our experimental conditions, AMY treatment decreased plasma Ca concentration since the highest calcemia was

measured in OVX rats, in which it was significantly higher than in SH or OVX+AMY rats, while no difference was observed between these two groups (Table 2). An increase in plasma Ca concentration measured in OVX rats in the present study may have resulted from increased bone resorption since estrogen deficiency stimulates osteoclastic activity. Osteoclasts contain estrogen receptors and respond to estrogen *in vitro* by decreasing resorptive activity (Oursler *et al.* 1991, 1994) and blocking the production of proinflammatory cytokines by bone marrow and bone cells (Pacifici 1998). The loss of estrogen at menopause creates an imbalance of osteoblastic and osteoclastic activities such that bone resorption outpaces bone formation (Dempster & Lindsay 1993, Horowitz 1993) leading to high turnover bone loss, post-menopausal osteoporosis and increased risk of fractures. Thus, in OVX rats, T-BMD and M-BMD (representative of trabecular bone) were lower than in SH or OVX+AMY rats, while no difference in D-BMD (representative of cortical bone) was observed between groups (Fig. 2). Such a lack of difference in D-BMD might partly explain while no difference in femoral Ca content was observed between groups (Table 2). Thus, AMY ameliorated bone loss more in trabecular than in cortical bone, perhaps explaining the absence of effect on femoral failure load in the present study (Fig. 1). The preventive effect of AMY treatment on OVX-stimulated trabecular bone resorption was reflected by urinary DPD excretion, lower in OVX+AMY than in OVX rats (Fig. 3).

AMY has been found to stimulate the proliferation of osteoblasts in a dose-dependent manner for concentrations as low as 10^{-11} M, and histomorphometric indices of bone formation are increased *in vivo* after the local injection of the peptide (Cornish *et al.* 1995, 1998*b*). In adult male mice given daily s.c. injections of AMY (10·5 μg, for 4 weeks) histomorphometric indices of bone formation increased 30–100% in the AMY-treated group, whereas resorption indices were reduced by 70%. Cortical width, tibial growth plate width, tibial length, body weight and fat mass were all increased in the AMY-treated group (Cornish *et al.* 1998*a*). Both an increase in osteoblastic activity and a decrease in bone resorption induced by AMY treatment is supported in the present study where plasma OC concentration was highest in the OVX+AMY group, in which urinary DPD excretion was simultaneously lower than in OVX rats (Fig. 3).

Romero *et al.* (1995) investigated the effects of AMY (100 pmol/100 g BW, s.c. for 19 days) on bone metabolism in normal and diabetic (streptozotocin-induced) rats. Analysis of the bone histomorphometry showed a low-turnover osteopenia in the diabetic animals. AMY administration resulted in a significant increase in bone volume in the normal rat, but was unable to significantly alter this parameter in diabetic rats. That study used a lower dose of AMY (about 0·3 μg/100 g BW vs 3 μg/100 g BW) during a shorter time (19 days vs 30 days), which might partly explain differences from the results reported here.

In conclusion, the present experiment indicates that, in OVX rats, AMY treatment partially inhibited estrogen deficiency-induced trabecular bone loss, by inhibiting resorption as well as by stimulating osteoblastic activity, as shown by decreased calcemia and urinary DPD excretion and increased plasma OC concentration measured in OVX rats treated with AMY. Thus, AMY appears as an attractive candidate as a therapeutic agent for osteoporosis.

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