Systemic administration of amylin increases bone mass, linear growth, and adiposity in adult male mice

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Systemic administration of amylin increases bone mass, linear growth, and adiposity in adult male mice. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E694–E699, 1998.—Amylin is a peptide hormone cosecreted with insulin from the pancreatic β-cells that can act as an osteoblast mitogen and as an inhibitor of bone resorption. The effects on bone of its systemic administration are uncertain. The present study addresses this question in adult male mice that were given daily subcutaneous injections of amylin (10.5 µg) or vehicle (n = 20 in each group) for 4 wk. Histomorphometric indices of bone formation increased 30–100% in the amylin-treated group, whereas resorption indices were reduced by ~70% (P < 0.005 for all indices). Total bone volume in the proximal tibia was 13.5 ± 1.4% in control animals and 23.0 ± 2.0% in those receiving amylin (P = 0.0005). Cortical width, tibial growth plate width, tibial length, body weight, and fat mass were all increased in the amylin-treated group. It is concluded that systemic administration of amylin increases skeletal mass and linear bone growth. This peptide has potential as a therapy for osteoporosis if its bone effects can be dissociated from those on soft tissue mass.

osteoporosis; bone metabolism; growth plate; obesity; osteoblasts

AMYLIN IS A 37-amino acid peptide hormone cosecreted with insulin from the β-cells of the pancreatic islets. It is structurally related to calcitonin, calcitonin gene-related peptide (CGRP), and adrenomedullin (sequence identities 13%, 43–49%, and 20%, respectively) (18). Since its discovery, much interest has focused on its possible roles in the pathogenesis and treatment of diabetes. However, it also has effects on bone metabolism, being a potent osteoblast mitogen (8, 23) and acting like calcitonin as an inhibitor of bone resorption. When administered locally over the calvariae of adult mice, it causes increased bone formation, reduced resorption, and a substantial increase in mineralized bone area after only 5 days of treatment (8). These findings suggest that it could contribute to the regulation of bone mass and that it is a potential therapy for osteoporosis. It is particularly attractive in the latter role because of its effects on both formation and resorption, a combination not shared by any other osteoporosis treatment. However, to be used in this role would require that amylin should increase bone mass when administered systemically. This issue is addressed in the present study.

METHODS

Experimental design. Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25–32 g were given daily subcutaneous injections (10.5 µg of rat amylin in 50 µl of water, or water alone) in the loose skin at the nape of the neck for 5 days/wk over 4 consecutive weeks. This amylin dose was chosen because it has previously been shown to produce significant effects on indices of carbohydrate metabolism (25). Animals were housed in a room maintained at 20°C on a 12:12-h light-dark cycle. They were fed diet 86 rodent pellets (New Zealand Stockfeed) ad libitum throughout the experiment. The weight of each animal was recorded at the start and end of the experiment. One day after the last injection, blood samples were taken by cardiac puncture under halothane anesthesia, and animals were killed by cervical dislocation. The study had the approval of the local institutional review board.

Histomorphometry. Indices of bone formation and resorption were assessed in the calvariae. This site was chosen because the method is well established in our laboratory and able to demonstrate clearly the effects of a variety of agents (8). Indices of bone volume were assessed in the tibiae, where the predominance of trabecular bone with its high surface-to-volume ratio results in larger changes in these measures than are seen elsewhere. Bones were dissected free of adherent tissue, and tibial lengths were recorded by measuring the distance between the proximal epiphysis and the distal tibiofibular junction with the use of an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Bones were placed in 10% phosphate-buffered Formalin for 24 h and then dehydrated in a graded series of ethanol solutions and embedded, undecalified, in methyl methacrylate resin. Tibiae were sectioned longitudinally through the frontal plane. Calvaria sections of 4 µm were cut from the base of the parietal bone, parallel and just anterior to the lambdoid suture. Sections were cut with the use of a Leitz rotary microtome (Leica Instruments, Nussloch, Germany) and a tungsten-carbide knife (Microknife Sharpening), mounted on gelatin-coated slides, and air-dried. They were stained with Goldner's trichrome and examined using an Olympus BX-50 microscope (Olympus Optical, Tokyo, Japan) that was attached to an Osteomeasure image analyzer (Osteometrics, Atlanta, GA).

Calvarial histomorphometric analyses were made in three adjacent fields from a single section, the first being one field from the central suture. Data were summed over the three sections and expressed per millimeter of section length. This results in >50% of a hemicalvariae being surveyed. Comparable fields were analyzed for all calvariae with the use of a ×20 objective lens. The precision of these histomorphometric measurements in our laboratory (expressed as coefficients of variation of paired measurements) are as follows: mineralized bone area 1.3%, osteoid area 6.9%, osteoblast perimeter 6.8%, osteoblast number 1.7%, eroded perimeter 6.7%, osteo-
clast perimeter 7.9%, osteoclast number <1.0%, calvarial length 0.2%, and calvarial width 1.7%.

Tibial histomorphometric analyses were made from three adjacent sections one-third of the way through the anterior/posterior depth of the proximal tibiae. All trabecular bone tissue in the secondary spongiosum was quantified in each section, and the parameters were derived using the formulas of Parfitt et al. (15). Cortical thickness was measured on both sides of the tibial shaft, 2.5 mm below the epiphyseal growth plate. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator (Cornish) who was blinded to the treatment group of each bone.

Fat mass estimations. Fat mass estimations were made from measurements of the body densities of the animals with the use of water displacement. Immediately after death, the mice were submerged headfirst to the base of the tail into a 250-ml measuring cylinder containing 150 ml of water, and the displacement volume was recorded. The fraction of body weight that was fat mass was calculated using a modification of the Siri equation for use in rodents (14). The coefficient of variation for repeated measures of fat mass is 7%.

Materials. Rat amylin was from Bachem California (Torrance, CA). To avoid losses when handling the peptide, an anti-static device was used to remove static electric charge from the peptide itself and from any containers in which it was placed (Zerostat 3, Discwasher, Reconton, Lake Mary, FL). The hydrochloride salt of the peptide was produced by dissolving it in 3 mM hydrochloric acid (10 µmol peptide in 50 µl) and leaving it for 1 h at room temperature before freeze-drying (model SVC 100H, Savant Instruments). Before use, it was redissolved in pure water with sonication (Soniprep 150, West Sussex, UK) and cooled on ice for 15 s, then stored at 4°C until required for injection. Amylin was dissolved for a minimum of 48 h before injection, since we have found that this increases the concentration of peptide measured by HPLC. The molecule is very adherent to glass, so only plastic containers are used in handling it.

Methyl methacrylate was purchased from Acros Organics NV (Geel, Belgium).

Statistical analysis. Data are presented as means ± SE. Where parameters have been measured more than once in each animal (e.g., cortical thickness), these values have been 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 all tests were two-tailed.

RESULTS

All three indices of bone formation were increased in the calvariae of the amylin-treated animals, being 30–100% above those seen in control mice (Fig. 1, top). In contrast, there was a substantial reduction in the three histomorphometric measures of bone resorption in the calvariae of the mice receiving amylin (Fig. 1, bottom). Consistent with these effects, trabecular bone volume of the tibiae was increased by 50% in animals receiving amylin (Figs. 2 and 3). This was contributed to by both an increased mean thickness of trabeculae and a decrease in their separation (Fig. 2). There was also a significant increase in the amount of cortical bone in the animals receiving amylin, as assessed by the cortical width in the tibiae of the amylin-treated animals (Fig. 2).

Amylin also influenced the tibial growth plate, increasing its width as well as increasing the total length of the tibiae (Fig. 4). Mean body weights at the start of the experiment were comparable in the two groups (28.6 and 29.3 g in the control and amylin groups, respectively), but the weight gain in the amylin-treated animals was significantly greater than that in the control group (8.1 ± 0.3 vs. 6.9 ± 0.2 g, P = 0.004). At the conclusion of the experiment, fat mass assessed from the density of the animals was significantly greater in the amylin-treated animals (2.9 ± 0.1 vs. 2.3 ± 0.2 g in the control animals, P = 0.01).
Blood taken at the time of death showed comparable serum concentrations of glucose (11.9 ± 0.5 and 12.1 ± 0.4 mmol/l in the control and amylin-treated groups, respectively), total calcium (2.24 ± 0.04 and 2.28 ± 0.03 mmol/l, respectively), and albumin (27.4 ± 0.3 and 27.2 ± 0.2 g/l, respectively) in the two groups.

DISCUSSION

The present data are the first to demonstrate changes in histomorphometric indices of bone formation and resorption after the systemic administration of amylin. They are striking in that the findings are entirely congruent with the actions of this peptide in isolated osteoclasts (1), in isolated osteoblasts (8), in bone organ culture (7, 16, 21), and after local administration of amylin in vivo (8). In each of these models, osteoblast activity is increased by this peptide and that of osteoclasts is reduced. This very consistent body of evidence establishes amylin as a peptide that uncouples bone formation from bone resorption, exerting opposite effects on these two activities and increasing bone mass as a result. Thus it has a unique profile of actions that raises the possibility that it might be a physiological regulator of bone mass [e.g., contributing to the high bone mass of hyperamylinemic states such as obesity (17)] and also makes it an attractive candidate as a therapy for osteoporosis. However, amylin might also have impact on bone indirectly, since it has effects on renal calcium handling (3) and increases circulating insulin-like growth factor I concentrations in goats (G. J. S. Cooper, personal communication).

The substantial increase in bone mass associated with amylin administration in the present studies is the outcome expected in light of the demonstrated changes in bone formation and resorption indices. In this regard, the present study is similar to that of Romero et al. (19), who found small increases in the bone mass of male rats in the absence of any changes in histomorphometric or biochemical indices of bone turnover. The more clear-cut result of the present study is possibly attributable to our use of a 100-fold larger dose of peptide. It is also possible that differences in peptide handling and dissolution may have contributed, since amylin passes only slowly into aqueous solution, is very adherent to plastic and glass surfaces, and (when in the powder form) is readily lost as a result of its high electrostatic charge. Careful attention to these issues has permitted us to effect a 10-fold increase in the apparent potency of amylin in in vitro experiments, and these practical issues may have contributed to the magnitude of the effects demonstrated in the present studies also. Other experimental differences such as the use of a different animal model from that used by Romero et al. may also have contributed to the more marked effects seen in the present study.

The dose of amylin used in the present studies is likely to be substantially supraphysiological. Young et al. (24) have measured circulating concentrations of rat amylin after subcutaneous injections of the same dose per kilogram as that used in the present study were given to rats. They found that the peak concentration was 1,000-fold above basal levels but that this fell rapidly. Extrapolation of their data suggests that basal levels would be reached within 4–6 h of the injection, suggesting that levels within the physiological range are likely to be present for much of each 24-h period.
Balanced against this is the size of the increments in bone mass demonstrated here, which are clearly much greater than would be seen with any physiological intervention. Thus the smaller postprandial excursions in amylin levels that occur normally could have a significant impact on bone mass over long periods of time.

As mentioned in the introduction, CGRP and calcitonin are both structurally related to amylin. It is, therefore, of interest to compare their effects on bone with those of amylin. Valentijn et al. (22) have recently shown partial prevention of postovariectomy bone loss in rats after daily injections of CGRP-α for 4 wk. The peptide dose used in those experiments was almost fourfold higher than that used in the present studies. The same group has presented a preliminary report that transgenic mice overexpressing the CGRP gene in bone have a 5% increase in distal femoral bone density at the age of 12 wk (2). Thus the present findings could represent a CGRP effect. However, we believe that this is unlikely, since we have directly compared amylin and CGRP with respect to their effects on osteoblast proliferation in vitro and on bone histomorphometry in vivo (8) and found amylin to be substantially more potent. Furthermore, the stimulation of osteoblast proliferation produced by $1 \times 10^{-8}$ M CGRP is completely blocked by the amylin receptor blocker amylin-(8—37) ($1 \times 10^{-10}$ M), whereas the CGRP receptor blocker CGRP-(8—37) is unable to completely block the effects of amylin in this model even when present in equimolar concentrations (unpublished observations). Burns and Kawase (4) recently compared the effects of amylin and CGRP on the development of mineralization in long-term osteoblast cultures and found amylin to be a more potent agent. These findings are consistent with the fact that the changes found in the present studies of amylin were achieved using lower doses of peptide than those of CGRP used by Valentijn et al. (22). Thus the osteogenic effects demonstrated in the present study are likely to be mediated by a receptor with a higher affinity for amylin than for CGRP, although the reduction in bone resorption may well be mediated by a calcitonin receptor. Calcitonin alone, however, is not able to reproduce the stimulation of bone formation observed in the present study (8, 20).

The effects of amylin on the width of the growth plate and on tibial length imply that the chondrocyte is also an amylin target cell. We have recently assessed this possibility directly in primary cultures of canine chondrocytes. These cells show increased thymidine incorporation and increased cell numbers after treatment for 24 h with amylin in concentrations of $1 \times 10^{-10}$ M and greater (unpublished observations). These changes in chondrocyte proliferation in response to amylin are comparable in magnitude to those we have previously described in osteoblasts with this peptide. The present findings are consistent with the fact that the changes found in the present studies of amylin were achieved using lower doses of peptide than those of CGRP used by Valentijn et al. (22). Thus the osteogenic effects demonstrated in the present study are likely to be mediated by a receptor with a higher affinity for amylin than for CGRP, although the reduction in bone resorption may well be mediated by a calcitonin receptor. Calcitonin alone, however, is not able to reproduce the stimulation of bone formation observed in the present study (8, 20).

![Fig. 3. Photomicrographs of proximal tibiae of mice treated with either vehicle (A) or amylin (B), demonstrating increase in trabecular bone volume associated with amylin treatment (original magnification, ×80).](image)

![Fig. 4. Effects of daily systemic administration of amylin for 4 wk on growth plate width and bone length in tibiae of normal adult male mice; n = 20 in each group. Data are means ± SE. *Significantly different from control, P < 0.004.](image)
findings suggest that hyperamylinemia may be associated with increased linear growth. Because nutrient intake results in amylin secretion, the regulation of chondrocyte function by amylin provides a pathway by which growth and the availability of the required substrates from food might be linked. This mechanism may have contributed to the progressive increases in the height of young adults over the last 100 years as nutrition has improved (11, 13).

An increase in the fat mass of amylin-treated animals has been predicted from its effects on intermediary metabolism (5). Amylin causes insulin resistance in the liver and in muscle (12) but not in adipocytes (6). Thus hyperamylinemia results in hyperinsulinemia, which, in turn, stimulates lipogenesis. In the context of potential treatment for osteoporosis, these effects on energy metabolism are undesirable. The intact amylin molecule is necessary for the peptide effects on carbohydrate metabolism. However, we have recently established that there are fragments of the amylin molecule that retain the capacity to stimulate osteoblast proliferation (10). We have also found that the structurally related peptide adrenomedullin shares amylin’s proliferative effects on osteoblasts in vitro and in vivo and that these can also be isolated to peptide fragments that lack the vasodilating effects of the parent molecule (9). Thus the potential exists to explore this family of peptides with a view to identifying those with optimal osteotropic effects but without deleterious actions on other tissues.

This research is potentially of substantial relevance to the therapy of osteoporosis. Bone mass can be increased either by the inhibition of bone resorption or by the stimulation of bone formation. The bisphosphonates provide a potent class of antiresorptive agents but can only reduce fracture rates by 50% and do not restore bone density to normal. Parathyroid hormone shows promise, although it probably needs to be administered together with an antiresorptive agent. One company has recently halted its research program in this area, apparently because of inadequate efficacy. Development of a second anabolic candidate, insulin-like growth factor I, has also stopped because of problems with hypoglycemia, and most of the other potential agents also have difficulties with toxicity arising from their actions on many tissues. Some of these have a propensity to stimulate the formation of woven rather than lamellar bone, and all are stimulators of bone resorption when given alone. The amylin-adrenomedullin family of peptides may be able to avoid many of these pitfalls, and, therefore, these peptides have a promising profile as potential anabolic factors for the treatment of osteoporosis. They merit examination in other models, including animals with postovariectomy bone loss.

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