Systemic administration of adrenomedullin(27–52) increases bone volume and strength in male mice

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Abstract

Adrenomedullin is a 52-amino acid peptide first described in a human phaeochromocytoma but since been found to be present in many tissues, including the vascular system and bone. Because of its structural similarity to amylin and calcitonin gene-related peptide, both of which have actions on bone cells, we have previously assessed the effects of adrenomedullin on the skeleton, and found that it increases osteoblast proliferation in vitro and bone formation following local injection in vivo. The present study carries this work forward by assessing the effects on bone of the systemic administration of a fragment of this peptide lacking the structural requirements for vasodilator activity. Two groups of 20 adult male mice received 20 injections of human adrenomedullin(27–52) 8·1 µg or vehicle over a 4-week period and bone histomorphometry and strength were assessed.

In the tibia, adrenomedullin(27–52) produced increases in the indices of osteoblast activity, osteoid perimeter and osteoblast perimeter (P<0·05 for both using Student’s t-test). Osteoclast perimeter was not affected. There was a 21% increase in cortical width and a 45% increase in trabecular bone volume in animals treated with adrenomedullin(27–52) (P<0·002 for both). Assessment of bone strength by three-point bending of the humerus showed both the maximal force and the displacement to the point of failure were increased in the animals treated with adrenomedullin(27–52) (P<0·03 for both). There was also a significant increase in the thickness of the epiphyseal growth plate. No adverse effects of the treatment were noted.

It is concluded that adrenomedullin(27–52) acts as an anabolic agent on bone. These findings may be relevant to the normal regulation of bone mass and to the design of agents for the treatment of osteoporosis.

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Introduction

Adrenomedullin is a 52-amino acid peptide first described in 1993 by Kitamura et al. (Kitamura et al. 1993). It was originally identified in a human phaeochromocytoma and has since been found to be present in normal adrenal medulla and in many other tissues, including the atra, ventricles, endothelial cells, lungs, brain, kidneys and bone (Ichiki et al. 1994, Montuenga et al. 1997, Hinson et al. 2000). It circulates in picomolar concentrations in both rats and man (Kitamura et al. 1994, Hinson et al. 2000). It is a potent vasodilator, acting directly on the renal, cerebral, mesenteric, pulmonary and systemic circulations, including the vascular supply of the skeleton (Kato et al. 1996). Its haemodynamic effects are probably mediated via receptors on vascular smooth muscle cells and possibly endothelial cells. Binding to a number of other tissues, including lung, heart, kidney and hypothalamus has also been demonstrated (Hinson et al. 2000). Adrenomedullin shows approximately 20% sequence identity with amylin and calcitonin gene-related peptide, and slightly less with calcitonin itself (Muff et al. 1995). All these peptides have an N-terminal ring created by a disulphide bond, and are amidated at their C-termini. Adrenomedullin differs from the others in that it has a linear N-terminal extension, consisting of 15 amino acids in the human and 13 in the rat.

Because of its structural similarity to amylin and calcitonin gene-related peptide, both of which have actions on bone cells, we assessed the effects of adrenomedullin on the skeleton (Cornish et al. 1997). At concentrations of 10−12 M and greater, adrenomedullin dose-dependently increased cell number and [3H]thymidine incorporation in cultures of foetal rat osteoblasts. This effect was also seen with adrenomedullin(15–52), adrenomedullin(22–52) and adrenomedullin(27–52) but not with adrenomedullin...
Adrenomedullin also increased $[^3]$H]thymidine incorporation into cultured neonatal mouse calvariae and phenylalanine incorporation into both isolated osteoblasts and calvariae (Cornish et al. 1997), and we have now also shown that it stimulates proliferation of primary cultures of human osteoblasts (J. Cornish, K. E. Callon & I. R. Reid, unpublished observations). Adrenomedullin injected daily for 5 days over the calvariae of adult mice increased indices of bone formation two- to threefold and increased mineralized bone area (Cornish et al. 1997).

This evidence of anabolic effects on the osteoblast suggests that adrenomedullin might have a role in the therapy of osteoporosis. However, adrenomedullin itself is unlikely to be suitable for such a role because it is a potent vasodilator. Our earlier investigations potentially addressed this problem by demonstrating osteotropic activity in adrenomedullin(27–52), a fragment that lacks the structural requirements for vasodilator activity (Santiago et al. 1995, Eguchi et al. 1994). The present study assesses the skeletal effects of daily systemic administration of this peptide in mice.

Materials and 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 (8.1 µg of human adrenomedullin(27–52) in 50 µl of water, or water alone) in the loose skin at the nape of the neck for 5 days/week over 4 consecutive weeks. This dose was chosen because the same molar dose (93 nmol/kg) of amylin in this model produces substantial effects on bone turnover and bone area (Cornish et al. 1998). Animals were housed in a room maintained at 20 °C on 12 h light : 12 h darkness cycles. They were fed diet 86 rodent pellets (New Zealand Stockfeed Ltd, Auckland, NZ) and allowed to feed ad libitum throughout the experiment. Each animal’s weight was recorded at the beginning and end of the experiment. The study had the approval of the local institutional review board.

Histomorphometry

Indices of bone formation and resorption, and bone volume were assessed in the proximal tibia. The tibiae were dissected free of adherent tissue and bone lengths were recorded by measuring the distance between the proximal epiphysis and the distal tibio-fibular junction using 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, undecalcified, in methylmethacrylate resin (Acros Organics, Geel, Belgium). Tibiae were sectioned longitudinally through the frontal plane. Sections (4 µm thick) were cut using a Leitz rotary microtome (Leica Instruments, Nussloch, Germany) and a tungsten carbide knife (Microknife Sharpening, UT, USA), then mounted on gelatin-coated slides, covered with plastic, pressed, and air-dried at 50 °C. They were stained with Goldner’s tri-chrome and examined using an Olympus BX 50 microscope (Olympus Optical Co. Ltd, Tokyo, Japan) which was attached to an Osteomeasure Image Analyzer (Osteometrics Inc., Atlanta, GA, USA).

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 spongiosa was quantified for bone volume in each section using a 10 × objective, and parameters were derived using the formulae of Parfitt et al. (1983). Parameters of bone formation and resorption were measured using a 20 × objective in all trabecular bone tissue in the secondary spongiosa in the middle of the three adjacent sections. The surfaces measured were those immediately adjacent to unmineralized matrix, those adjacent to osteoblasts, and those adjacent to osteoclasts (osteoid, osteoblast, and osteoclast perimeters respectively). Perimeters were expressed per section. Cortical width 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 (J.C) who was blinded to the treatment group of each bone. The precisions of these histomorphometric measurements in our laboratory (expressed as coefficients of variation of paired measurements) are as follows: mineralized bone area 1-3%, osteoid perimeter 6-9%, osteoblast perimeter 6-8%, osteoclast perimeter 7-9%, and width measurements 1-7%.

Mechanical strength of the humeri

One humerus from each animal was used for mechanical strength estimations by three-point bending tests. Each bone was dissected free of soft tissue and tested on a MTS 858 Bionix Testing Machine (MTS Systems Corporation, Minneapolis, MN, USA). Samples were tested at room temperature with a support span of 10 mm. Load was applied at a constant deformation rate of 2 mm/min with a force application at the midpoint of the bone. Load–deformation curves were recorded and displacement values (a measurement of how much the bone bends from the time that the force is applied until its final failure point) and the maximal force were obtained directly from the curve and expressed in millimetres (mm).

Fat mass estimations

Fat mass estimations were made from measurements of the animals’ body densities calculated from water displacement. Immediately after death the mice were submerged.
head-first to the base of the tail into a 250 ml measuring cylinder containing 150 ml of water and the displacement volume recorded. The fraction of body weight that was fat mass was calculated using a modification of the Siri equation for use in rodents (Muscaritoli et al. 1993). The coefficient of variation for repeated measures of fat mass is 7%.

Biochemistry

Serum calcium and albumin were measured using an Hitachi 917 Analyser (Roche Diagnostics, Mannheim, Germany), serum intact mouse parathyroid hormone using an ELISA kit (Immutopics, San Clemente, CA, USA), and serum 25-hydroxyvitamin D by RIA (DiaSorin, Stillwater, MN, USA).

Materials

Human adrenomedullin(27–52) was synthesized on methylbenzhydrylamine resin using standard solid-phase procedures, and cleaved with hydrogen fluoride/anisole. Crude materials were purified by gel filtration on Sephadex columns in 50% acetic acid followed by gradient elution on C18 silica using acetonitrile/0·1% trifluoroacetic acid eluants. Homogeneity of the final peptide was assessed by thin-layer chromatography, analytical HPLC, amino acid analysis and matrix-assisted laser-desorption-ionization mass spectroscopy. Purity was >98%.

To avoid losses when handling the peptide, an anti-static device (Zerostat 3, Discwasher, Reconton Corp., Lake Mary, FL, USA) was used to remove static electric charge from the peptide itself and from any containers in which it was placed. The hydrochloride salt of the peptide was produced by dissolving it in 3 mM hydrochloric acid and leaving it for 1 h at room temperature before freeze-drying (Savant Instruments, model SVC 100H, New York, NY, USA). Before use it was re-dissolved in pure water with sonication (Soniprep 150, West Sussex, UK) and cooling on ice for 15 s, then stored at 4 °C until required for injection. Adrenomedullin(27–52) was dissolved for a minimum of 48 h prior to injection since we have found that this increases the concentration of peptide in solution, measured by HPLC. The molecule is very adherent to glass so only plastic containers were used in handling it.

Statistical analysis

Data are presented as means ± s.e.m. 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, except for the bone strength data which were analysed using the Wilcoxon rank sum test for independent groups. This was necessary because those data were not normally distributed. A 5% significance level was maintained and all tests were two-tailed.

Results

Adrenomedullin(27–52) produced increases in indices of osteoblast activity, osteoid perimeter increasing from 6·7 ± 0·4 to 8·0 ± 0·4 mm, and osteoblast perimeter from 3·2 ± 0·2 to 4·0 ± 0·2 mm (Fig. 1). In contrast, osteoclast perimeter was not different between the groups (control 0·25 ± 0·02, adrenomedullin(27–52) 0·29 ± 0·02). Accompanying these changes was a 21% increase in cortical width (from 0·144 ± 0·004 mm in control animals to 0·174 ± 0·008 mm in those receiving adrenomedullin (27–52)) and a 45% increase in trabecular bone volume (from 13·6 ± 1·0% in the control animals to 19·8 ± 1·2%
Serum calcium concentrations were higher in the adrenomedullin(27–52) group at the end of the treatment period, though still within the normal range (adrenomedullin 2.49 ± 0.03 mmol/l, control 2.37 ± 0.02 mmol/l, \(P<0.005\)). Parathyroid hormone (46 ± 6 pg/ml vs 38 ± 6 pg/ml) and 25-hydroxyvitamin D (23 ± 1 µg/l vs 24 ± 1 µg/l) were not different between the groups.

During the 4 weeks of the study, body weight increased from 27.8 ± 0.4 g to 32.8 ± 0.5 g in the control animals, and from 27.7 ± 0.5 g to 33.2 ± 0.6 g in those treated with adrenomedullin(27–52). Fat masses at the end of the study were 2.43 ± 0.13 g and 2.08 ± 0.14 g in the control and adrenomedullin(27–52) animals, respectively. These results were not significantly different between the groups. All animals were apparently in good health throughout the study, as judged by weight gain, coat condition and behaviour.

### Discussion

The present findings confirm those of our earlier work, that adrenomedullin has an anabolic effect on osteoblasts. This has now been demonstrated in isolated rodent and human osteoblasts, in tissue culture of neonatal mouse calvariae, following local injection over the calvariae of adult mice \textit{in vivo}, and now following systemic administration. There is evidence of effects on cortical and trabecular bone, both of which are substantial, and of an effect on integral bone strength. \textit{In vitro}, the effects on osteoblast proliferation of intact adrenomedullin and those of its C-terminal peptide studied here are indistinguishable (Cornish et al. 1997). \textit{In vivo} the effects on osteoblasts of intact adrenomedullin and the related peptide, amylin, are also comparable (Cornish & Reid 1999). Thus, it is not surprising that the increase (45%) in trabecular bone volume produced by adrenomedullin(27–52) in the present study is very similar to that resulting from amylin treatment in the same animal model (50%) (Cornish et al. 1998). This evidence of an anabolic effect of adrenomedullin, together with the observations that the mRNA for adrenomedullin is strongly expressed in primary osteoblast cultures and in a clonal osteoblast cell line (UMR 106) (Naot et al. 2001), as well as in the skeleton of rodent embryos (Montuenga et al. 1997), suggest that adrenomedullin may play a physiological role in the development and maintenance of bone.

The increase in serum calcium in the animals treated with adrenomedullin(27–52) is small and unlikely to be biologically significant. It probably results from the direct effect of adrenomedullin(27–52) to increase bone turnover. In the present study, there was not a statistically significant increase in histomorphometric measures of bone resorption, though the trend in osteoclast perimeter was upward. However, in our earlier study of local injection of adrenomedullin over the calvariae of adult

![Figure 2](image_url)

\textbf{Figure 2} Effects of daily systemic administration of adrenomedullin(27–52) for 4 weeks on histomorphometric indices of bone volume in the tibiae of normal sexually mature male mice. \(n=20\) in each group. Data are means ± S.E.M. *, significantly different from control, \(P<0.002\).
mice, there was a significant increase in bone resorption (Cornish et al. 1997).

The observation that the thickness of the epiphyseal growth plate is increased with adrenomedullin(27–52) treatment is novel and of great interest. It parallels a similar recent finding with amylin (Cornish et al. 1998), suggesting that this might be an integral property of this family of peptides. It is likely to be mediated by a direct effect on cartilage, since we have demonstrated stimulation of proliferation of primary cultures of canine and human articular chondrocytes by both amylin and adrenomedullin (manuscript in preparation). The similar embryological origin of chondrocytes and osteoblasts suggests that similar receptors and post-receptor pathways might mediate these effects.

The nature of these pathway(s) remains to be resolved. We have recently demonstrated that the mRNAs for three putative adrenomedullin receptors are expressed in
primary cultures of rat osteoblasts (Naot et al. 2001), and Montuenga previously demonstrated that one of these was present in rodent embryos. In other cell types, these receptors are coupled to adenylyl cyclase and are thought to mediate the vasodilator properties of adrenomedullin. Adrenomedullin action on bone, however, is associated with only minimal changes in cyclic AMP concentrations (J Cornish, K E Callon & I R Reid, unpublished observations) and the osteotropic effect persists in adrenomedullin fragments which are inactive on vascular smooth muscle cells. In addition, adrenomedullin activity in bone is associated with activation of mitogen-activated protein kinase (Grey et al. 1999), and also appears to require the presence of a functional insulin-like growth factor-I receptor (Cornish et al. 1999). Thus, it is possible that the anabolic effects of adrenomedullin on osteoblasts may be mediated by a mechanism which is quite distinct from many of its other biological effects. A fuller understanding of the receptor(s) involved and the associated post-receptor events will be necessary for a full appreciation of the role of this peptide in normal skeletal homeostasis, and to any harnessing of these effects for therapeutic use.

The present findings are potentially of relevance to the development of therapies for osteoporosis. There is a great need for a safe and effective agent which promotes osteoblast growth and thus produces sustained increases in bone mass. Such a pharmaceutical would complement the variety of anti-resorptive therapies that are currently available for managing this condition. The present demonstration of anabolic activity in adrenomedullin(27–52) is, therefore, of great interest, particularly so since this peptide fragment, unlike its parent hormone, is not vasodilatory. These findings point to a bone-specific activity that may be able to be further refined with a view to its therapeutic use. The assessment of smaller fragments of the adrenomedullin molecule to determine the minimal requirements for activity on osteoblasts, and the optimisation of this activity through the development of further peptide analogues, are both avenues of research that now require exploration. It is ultimately possible that non-peptide analogues may be able to be developed which mimic this activity of adrenomedullin, but which would have the advantage of being simple to manufacture and being suitable for oral administration. Ultimately, this work will need to be translated into human studies before its therapeutic relevance can be assessed.

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**Figure 4** Effects of daily systemic administration of adrenomedullin(27–52) for 4 weeks on mechanical strength of the humerus, assessed by maximal force (F\textsubscript{max}) and displacement values from load–deformation curves obtained from three-point bending tests. n=20 in each group. Data are means ± s.e.m. *, significantly different from control, P<0.03.

**Figure 5** Effects of daily systemic administration of adrenomedullin(27–52) for 4 weeks on the thickness of the epiphyseal growth plate in the tibiae of normal sexually mature male mice. n=20 in each group. Data are means ± s.e.m. *, significantly different from control, P=0.003.
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