Molecular and Cellular Endocrinology 365 (2013) 309–315

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Amylin exerts osteogenic actions with different efficacy depending on the diabetic status

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ARTICLE INFO

Article history:
Received 10 September 2012
Received in revised form 17 October 2012
Accepted 12 November 2012
Available online 21 November 2012

Keywords:
Bone
Amylin
Diabetes
Insulin-resistance

ABSTRACT

Amylin displays osteogenic features, but its role in diabetic osteopenia is unclear. We examined the possible osteogenic action of amylin infusion for 3 days into fructose-induced insulin-resistant (IR) and streptozotocin-induced type 2 diabetic (T2D) and normal (N) rats. Amylin failed to affect glycaemia or parathyroid hormone levels in any group, but reduced hyperinsulinemia in IR rats. In N rats, amylin increased bone formation rate and reduced osteoclast surface and erosive surface in the femoral metaphysis, and increased osteoprotegerin (OPG)/receptor activator of NFκB ligand (RANKL) mRNA ratio in the tibia. In T2D rats, amylin normalized trabecular structure parameters and increased osteoblast number and osteocalcin (OC) expression in long bones. In contrast, in IR rats, no apparent osteogenic effect of amylin in the femur was observed, although both OC and OPG/RANKL ratio were increased in the tibia. Our findings demonstrate a different osteogenic efficacy of amylin in two diabetic settings.

1. Introduction

Amylin is a hormone synthesized by the pancreatic islet β-cell, from which it is released together with insulin in response to food intake and other stimuli (Kahn et al., 1990; Novials et al., 1993). Amylin regulates fuel metabolism in association with other metabolic, endocrine and neural influences (Cooper, 1994). This hormone shows the characteristics of a peripheral satiety signal (Barth et al., 2003; Lutz, 2005; Riediger et al., 2004), primarily acting at the hindbrain and the rostral central nervous system (Creutzfeldt, 2001; Young et al., 1996), after amylin binding to specific receptors in the area postrema (Christopoulos et al., 1999). Amylin decreases body weight gain and reduces adiposity (Roth et al., 2008). In addition, recent *in vitro* studies also indicate that amylin can restore the altered glucose homeostasis in target tissues such as adipose tissue and liver in states of insulin-resistance and type 2 diabetes mellitus (Moreno et al., 2011). On the other hand, an impaired amylin regulation appears to occur associated with hypertension in the setting of the metabolic syndrome (Kailasam et al., 2000). In fact, amylin analogs are now envisioned as putative therapeutic agents not only in obesity (Dunican et al., 2010) but also in obesity-related syndromes such as diabetes mellitus (Lebovitz, 2010; Osaka et al., 2008).

As mentioned above, amylin is co-secreted with insulin after a meal. It is well known that insulin as well as other hormones, including those of incretin character, which are postpandrially released, can act as anabolic stimulus to the skeleton (Clowes et al., 2005). In this scenario, the concerted action of these physiological factors together with diet-supplied substrates would positively contribute to bone formation. Therefore, it makes sense that amylin might act in concert with other postprandial factors in the physiological control of bone maintenance. Only a few studies have yet been performed to address this hypothesis. It was shown that *s.c.* injection of amylin at nM concentration into mouse calvariae for 5 days increased osteoblast numbers and osteoblastic activity; meanwhile, amylin inhibited osteoclast-mediated bone resorption

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http://dx.doi.org/10.1016/j.mce.2012.11.013

(Cornish et al., 1995, 1998b). Moreover, systemic injection of amylin into adult normal mice increased bone mass (Cornish et al., 1998a). Several *in vitro* reports have shown that amylin stimulates osteoblastic cell proliferation (Cornish et al., 1995, 1998b; Villa et al., 1997), although some conflicting results have also been published in this regard (Ellegaard et al., 2010).

Amylin mimics many if not all the actions of glucagon-like peptide (GLP-1), an incretin with both insulinotropic and insulin-independent antidiabetic properties, affecting glucose and energy metabolism (Creutzfeldt, 2001; Sancho et al., 2005; Valverde et al., 1994). However, recent studies performed in normal and type 1 diabetic (T1D) patients (Asmar et al., 2010), and also in a nonhuman primate model (Bello et al., 2010), have suggested that the common effects of GLP-1 and amylin on gastric emptying, food intake and glucagon release are exerted in an independent manner from each other, and point to the potential advantage for the combined GLP-1 and amylin therapy (see Roth et al., 2012 for review).

Recently, continuous infusion for 3 days of either GLP-1 or its analog exendin-4 into normal rats was shown to have a dual effect on bone turnover: inhibiting bone resorption through an increased osteoprotegerin (OPG)/receptor activator of NFκB ligand (RANKL) ratio, and increasing bone formation as suggested by an augmented expression of osteocalcin (OC) in the tibia (Nuche-Berenguer et al., 2009, 2010). Moreover, these peptides were able to improve the deleterious trabecular structure in the long bones using well characterized insulin resistant (IR) or type 2 diabetic (T2D) rat models (Nuche-Berenguer et al., 2009, 2010). Thus, the question arises as to whether amylin might be as efficient as GLP-1 at correcting the altered bone quality in the setting of osteopenia as occurs in the latter diabetic states (Blakytny et al., 2011). In spite of the scanty experimental evidence in this respect, such assumption has recently been proposed for some chemically modified analogs of amylin (Kowalczyk et al., 2012). In view of these considerations, in the present study we aimed to unravel the putative osteogenic action of amylin in diabetic conditions.

2. Material and methods

2.1. Reagents

Rat amylin and insulin (Bachem AG, Bubendorf, Switzerland); streptozotocin (STZ), demeclocycline, and TRI Reagent™ for RNA isolation (Sigma–Aldrich, St. Louis, MO, USA); high-capacity cDNA reverse transcription kit and Taqman Universal PCR master mix as well as TaqMan probes for rat osteocalcin (OC, Rn00566386_g1), osteoprotegerin (OPG, Rn00563499_m1), receptor activator of NF-κB ligand (RANKL, Rn00569289_m1), and 18S (4319413E), from Applied Biosystems (Foster City, CA, USA); methylmethacrylate (Merck Pharma Química, Barcelona, Spain). All other commonly used chemicals were from Sigma–Aldrich or Merck.

2.2. Animals

Male Wistar rats, kept on a standard pellet diet (Panlab, Barcelona, Spain) and tap water *ad libitum*, were used. The IR model was prepared in 5 weeks old animals by chronic feeding for 8 weeks a standard chow combined with D-fructose dissolved in the drinking water at 20% concentration (Cancelas et al., 2008). To generate the T2D model, a single dose of STZ dissolved in 50 mM sodium citrate buffer, pH 4.5, was intraperitoneally injected (100 μg/g body weight) on the day of birth (Iwase et al., 1987; Portha et al., 1979). After 8 weeks, those animals showing a glucose disappearance constant below 2.5×10^{-2}/min during an *i.v.* glucose tolerance test (0.05 mg glucose/g body weight/30 s) were selected. All rats of the two experimental models, and also normal (N) rats as control group, had the same age (13 weeks old) at the start of the study protocol. Animal housing and protocols were approved by the Animal Use Committee of the IIS-Fundación Jiménez Díaz.

2.3. Experimental design

N, IR and T2D rats were subjected to a 3-day treatment with amylin dissolved in saline solution, at 30 pmol/kg/h, or saline alone (controls), by continuous infusion through a subcutaneously implanted osmotic pump (Alzet 1003D; Alza, Palo Alto, CA, USA). Some rats from each experimental group were intraperitoneally injected with demeclocycline (20 mg/kg) at days 13 and 4 before sacrifice. Just before (basal) and during these treatments (at day 2), blood samples were collected from the rat tail for measuring glucose, insulin, amylin and parathyroid hormone (PTH) in plasma. Afterwards, the animals were stunned and sacrificed by a sharp blow to the head. The tibiae were collected and immediately frozen at −70° C until total RNA isolation; the femurs from some rats were also collected, stripped of soft tissue, and kept in 70% ethanol for performing analysis of bone mineral density (BMD), bone mineral content (BMC) and bone histomorphometry. No significant changes in body weight were detected in the three groups of rats throughout the study.

2.4. Plasma measurements

Glucose was determined by the glucose oxidase method (Glucose analyzer 2; Beckman, Galway, Ireland); insulin was measured by radioimmunoassay (Herbert et al., 1956), using rat insulin as standard and a guinea pig polyclonal anti-insulin antiserum (GP-25) developed in our laboratory (Valverde et al., 1988). Amylin was determined by ELISA (Millipore Co., MA, USA), and PTH was evaluated using a PTH (1–34) immunoassay kit (Peninsula Laboratories, San Carlos, CA).

2.5. Isolation of total bone RNA and real time PCR

The tibiae were individually powdered with a cold steel mortar and pestle, and then mixed with TRI Reagent™ for total RNA extraction following manufacturer's instructions. Total RNA concentration and purity were estimated by spectrophotometric absorption at 260 nm and 280 nm, respectively, in an aliquot volume of each sample (Arnés et al., 2008). cDNA was synthesized from 4 μg of total bone RNA using avian myeloblastosis virus reverse transcriptase with random hexamer primers. PCR amplification was subsequently carried out in a StepOne system (Applied Biosystems), in triplicate for each sample, in a total volume of 20 μl containing 3 μl of cDNA, 0.5 μl of the respective probe and 10 μl of TaqMan Universal PCR Master Mix; the conditions of amplification and detection −50 cycles/110 min – were as described (Nuche-Berenguer et al., 2009). For each rat, the gene expression was normalized with that of the housekeeping gene 18S, and expressed as $2^{-\Delta\Delta Ct}$.

2.6. Dual-energy X-ray absorptiometry (DEXA) and bone histomorphometry

BMD and BMC in the total femur of each rat were determined by DEXA using a PIXImus densitometer (GE Lunar, Madison, WI, USA). For bone histomorphometry analysis, the femoral bone specimens were dehydrated in graded ethanols and embedded in methylmethacrylate. Sagittal longitudinal sections (7 mm thick) of the femur were obtained with a rotation microtome for hard materials (Leica RM2255, Leica Microsystems, Nussloch, Germany), and then, they were stained with Von Kossa and Goldner's Trichrome as recently described (Nuche-Berenguer et al., 2011) for evaluation of the following static parameters in the proximal metaphysis: