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Chronic administration of Glucagon-like peptide-1 receptor agonists improves trabecular bone mass and architecture in ovariectomised mice

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ABSTRACT

Some anti-diabetic therapies can have adverse effects on bone health and increase fracture risk. In this study, we tested the skeletal effects of chronic administration of two Glucagon-like peptide-1 receptor agonists (GLP-1RA), increasingly used for type 2 diabetes treatment, in a model of osteoporosis associated bone loss and examined the expression and activation of GLP-1R in bone cells. Mice were ovariectomised (OVX) to induce bone loss and four weeks later they were treated with Liraglutide (LIR) 0.3 mg/kg/day, Exenatide (Ex-4) 10 μg/kg/day or saline for four weeks. Mice were injected with calcein and alizarin red prior to euthanasia, to label bone-mineralising surfaces. Tibial micro-architecture was determined by micro-CT and bone formation and resorption parameters measured by histomorphometric analysis. Serum was collected to measure calcitonin and sclerostin levels, inhibitors of bone resorption and formation, respectively. GLP-1R mRNA and protein expression were evaluated in the bone, bone marrow and bone cells using RT-PCR and immunohistochemistry. Primary osteoclasts and osteoblasts were cultured to evaluate the effect of GLP-1RA on bone resorption and formation in vitro. GLP-1RA significantly increased trabecular bone mass, connectivity and structure parameters but had no effect on cortical bone. There was no effect of GLP-1RA on bone formation in vivo but an increase in osteoclast number and osteoclast surfaces was observed with Ex-4. GLP-1R was expressed in bone marrow cells, primary osteoclasts and osteoblasts and in late osteocytic cell line. Both Ex-4 and LIR stimulated osteoclastic differentiation in vitro but slightly reduced the area resorbed per osteoclast. They had no effect on bone nodule formation in vitro. Serum calcitonin levels were increased and sclerostin levels decreased by Ex-4 but not by LIR. Thus, GLP-1RA can have beneficial effects on bone and the expression of GLP-1R in bone cells may imply that these effects are exerted directly on the tissue.

1. Introduction

Osteoporosis, osteoarthritis, joint deformities and fractures affect a large proportion of the elderly population and represent important causes of morbidity. The incidence of these conditions is significantly increased in the presence of types 1 and 2 diabetes mellitus (DM) [1–3]. Clinical data indicate that the bone of diabetic patients is fragile and of poor quality, despite a bone mineral density (BMD) often normal. Circulating levels of sclerostin, a negative regulator of bone formation produced by osteocytes, are elevated in type 2 diabetic patients [4]. Although the mechanisms leading to the poor bone strength and quality in DM patients are not entirely known, accumulation of advanced glycation end products, changes in collagen cross-linking and suppression of bone turnover are significant contributors [5,6]. In addition to the effect of DM itself on the bone, adverse impacts on bone health of some anti-diabetic drugs have been reported, such as thiazolidinediones (TZDs) that increase fracture risk [7]. In contrast, metformin, another widely prescribed anti-diabetic drug, inhibits the bone loss induced by ovariectomy (OVX) in rats [8] and reduces the risk of fracture in DM patients [9]. However, our recent studies showed no beneficial effect of metformin on bone mass and fracture healing in rodents [10].

Incretin hormones such as Glucagon-like-peptide 1 (GLP-1), GLP-2 and glucose-dependent insulinotropic peptide (GIP) are peptides secreted in the gastrointestinal tract in response to ingestion of nutrients with insulin-independent anti-diabetic properties [11]. Following its secretion from the intestinal L-cells, GLP-1 binds to its receptor (GLP-1R) on pancreatic β-cells to stimulate insulin secretion [12]. As GLP-1 is quickly degraded in the circulation by the ubiquitous protease dipeptidyl peptidase-IV (DPP-4), Glucagon-like-peptide 1 receptor agonists (GLP-1RA) with an extended half-life by virtue of their resistance to degradation by DPP-4 have been developed for clinical use, including Exendin-4 (Ex-4) and Liraglutide (LIR) [13,14]. GLP-1RA, administered either as a monotherapy or in combination with other existing oral anti-diabetic

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drugs [15], are now increasingly used for the treatment of DM, as they provide additional extra-glycaemic effects, such as weight loss [16].

Several studies have demonstrated that GLP-1RA affect bone turnover [17,18]. GLP-1 has been shown to indirectly inhibit bone resorption via stimulation of calcitonin production induced by its binding to the GLP-1R in thyroid C cells [19]. Accordingly, mice with deletion of pancreatic GLP-1R develop cortical osteopenia and show increased bone resorption through a calcitonin-dependent pathway [20]. Another study showed a similar positive effect of GLP-1R activation on bone strength and quality, as mice lacking GLP-1R showed significantly impaired mechanical properties, a decrease in cortical thickness and bone outer diameter and a reduction in the maturity of the collagen matrix [21]. Similarly, double incretin receptor knock-out (DIRKO) mice exhibit dramatic and profound alterations of bone microarchitecture and strength, confirming the importance of incretin hormones in the regulation of bone quality [22]. 3 day infusion or daily injections of GLP-1RA for 3 days were shown to be anabolic in the bone of normal, insulin-resistant (IR) and T2DM rodent models [23–27]. In addition, it was recently demonstrated that long-term treatment with the GLP-1RA Ex-4 prevents osteopenia in aged ovariectomised rats, a model of bone loss that mimics osteoporosis [28]. It is, however, unclear whether the mechanism of action of GLP-1RA in the bone is direct through a functional GLP-1R expressed by bone cells or indirect via an increase in calcitonin production. Furthermore, there are inconsistencies in the literature regarding the expression of GLP-1R in the bone and thus the basis for direct skeletal effects of GLP-1. While previous in vivo studies indicate indirect effects of GLP-1 on the skeleton via a calcitonin-dependent pathway [20], it has recently been shown that mouse osteoblast-like MC3T3-E1 cells express a functional receptor for GLP-1, different from the cAMP-linked GLP-1R expressed in the pancreas, suggesting a possible direct skeletal action of GLP-1 [29,30]. In contrast, expression of the pancreatic-type GLP-1R mRNA was identified in human osteoblastic cell lines, although its expression varied between them [31]. The presence of pancreatic GLP-1R has also been reported in osteocytic MLO-Y4 cells and osteocytes in rat femurs [26], as well as in mesenchymal stem cells [32]. GLP-1R expression is increased during osteogenic differentiation of adipose derived stem cells (ADSCs), suggesting that GLP-1R activation may contribute to osteogenesis [33].

In this study, we wanted to examine the skeletal effects of GLP-1RA in a model of osteoporosis-associated bone loss. We investigated the effects of chronic administration of two different GLP-1RA on bone mass, architecture, cellular activities in situ and production of calcitonin and sclerostin in ovariectomised mice. We also aimed to determine if GLP-1RA can directly affect bone cell function in vitro through a receptor expressed in bone cells.

2. Material and methods

2.1. Animals and study design

Thirty 12 week old female C57Bl/6NCrl mice were obtained from Charles River Laboratories, Inc. (Margate, UK). Mice were all ovariectomised [34] and four weeks later, divided randomly into three treatment groups: one group (n = 10) was treated with 10 μg/kg/day Exenatide (Bachem) dissolved in saline, the second was treated with 0.3 mg/kg/day Liraglutide (Bachem) dissolved in saline and the last group received saline (control). All treatments were administered by daily subcutaneous injections for 4 weeks. At days 6 and 3 prior to euthanasia, mice were intraperitoneally injected with calcein (20 mg/kg) and alizarin red complexone (30 mg/kg) (Sigma-Aldrich), respectively, to label bone-mineralising surfaces in the trabecular bone. At the end of the experiment, mice were sacrificed, the serum collected for sclerostin and calcitonin measurements, and right tibiae dissected for micro-CT analysis and left tibiae for bone histomorphometry. The success of ovariectomy was confirmed by observation of uterine atrophy during dissection. All animal experimentation procedures were performed in compliance with local ethical committee and Home Office Project Licence under the auspices of the UK Animals (Scientific Procedures) Act 1986.

2.2. Micro-CT analysis of tibiae

Right tibiae were fixed in 10% neutral-buffered formalin for 24–72 h and stored in 70% ethanol at 4 °C. They were then scanned using high-resolution (5 μm pixel size) micro-computed tomography (micro-CT) (skyScan-1172/F BRUKER, Belgium), as previously described [35]. After scanning, the data was reconstructed with NRecon version 1.6.4.1 (NRecon®). Trabecular and cortical bone areas were analysed with CT-Analyser (CTAn) version 1.11.10.0. For analysis of the trabecular bone in proximal metaphyses, the cortical shell was excluded by operator-drawn regions of interest and 3D algorithms were used to determine the relevant parameters, which included: bone volume percentage (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), structure model index (SMI), trabecular pattern factor (Tb.Pf), trabecular separation (Tb.Sp) and degree of anisotropy (DA). Analysis of the cortical bone in midshaft diaphyses was performed using a 0.49 mm long segment (or 100 tomograms) at 37% of tibial length from its proximal end. Cortical bone parameters consisted of: tissue area (Tt.Ar), tissue perimeter (Tt.Pm), bone area (Ct.Ar), eccentricity (Ecc), moment of inertia (MMI polar) and cross-sectional thickness (Ct.Th).

2.3. Bone histomorphometry

Left tibiae were fixed in 4.5% formaldehyde for 2 days at 4 °C, dehydrated in acetone for 24 h and embedded in methyl methacrylate (MMA) at low temperature to preserve enzymatic activity [36]. Unstained 8-μm-thick longitudinal sections were used for fluorescence microscopy to assess mineral apposition rate (MAR, μm/day). Area of mineralising surfaces was expressed as alizarin red-labelled surfaces per bone surfaces (MS/BS, %) and the bone formation rate was calculated as MS/BS × MAR (BFR/BS, $\mu m^3/\mu m^2$/day). Alternatively, sections were stained for tartrate-resistant acid phosphatase (TRAP) (Leucognost® SP; Merck, Germany) and counterstained with Mayer's haematoxylin solution. Histomorphometric parameters were measured on the trabecular bone of the metaphysis, on a region of interest consisting of 2 mm width below the growth plate. Measurements were performed using image analysis software (Tablet'measure; Explora Nova, La Rochelle, France). Histomorphometric parameters were reported in accordance with the ASBMR Committee nomenclature [37].

2.4. Immunohistochemistry for GLP-1R

Adult wild-type C57BL/6 mouse femurs were fixed in 10% neutral buffered formalin, cast in paraffin and sectioned at 6 μm. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol (10 min), and tissue sections were permeabilised in 1% Sodium Dodecyl Sulphate (SDS) in Tris Buffered Saline (TBS) for 5 min and blocked with 3% BSA in 20% goat serum. Samples were incubated overnight at 4 °C with rabbit polyclonal antibody anti-GLP-1R (1 μg/ml) (ab39072, Abcam, Cambridge) and rabbit IgG for control sections (Vector labs). Sections were washed with TBS-Tween (TBST) and incubated in biotinylated secondary antibody (goat anti-rabbit; 1:300) for 1 h at room temperature in TBST containing 1% BSA. Sections were washed with TBST, and incubated with Avidin Biotin Complex (ABC) to amplify the target antigen signal before a second wash with TBST and a final incubation with DAB (3, 3′-diaminobenzidine). Sections were counterstained briefly with haematoxylin and imaged using light microscopy.