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

M. Pereira [a,*], J. Jeyabalan [a], C.S. Jørgensen [a], M. Hopkinson [a], A. Al-Jazzar [a], J.P. Roux [b], P. Chavassieux [b], I.R. Orriss [a], M.E. Cleasby [a], C. Chenu [a]

[a] Department of Comparative and Biomedical Sciences, Royal Veterinary College, London NW1 0TU, UK
[b] INSERM UMR1033 and Université de Lyon, Lyon, France

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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

* Corresponding author at: Royal Veterinary College, Department of Comparative and Biomedical Sciences, Royal College Street, London NW1 OTU, UK.
E-mail address: mpereira@rvc.ac.uk (M. Pereira).

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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.

2.5. Cell culture

2.5.1. Osteocytic cell line MLO-A5

Murine pre-osteocytic cells (MLO-A5) were cultured in minimal essential medium (MEM), supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.5.2. Osteocytic cell line IDG-SW3

The mouse osteoblastic-late-osteocytic cell line IDG-SW3 was kindly provided by Prof Linda Bonewald and cultured as previously described [38] in MEM containing L-glutamine supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. 50 U/ml of recombinant Mouse Interferon-gamma (INF-γ) (Invitrogen) was added to induce expression of the SV40 large tumour antigen and maintain proliferation of this cell line. The IDG-SW3 cells were expanded on rat tail collagen type 1-coated plates (Becton Dickson Bioscience) at 40,000 cells/cm^2 at 33 °C. Osteogenesis was induced by replacing medium at confluence with fresh growth medium supplemented with 50 mg/l ascorbic acid and 4 mM β-glycerophosphate without IFNγ at 37 °C. Cells were maintained for 30 days in osteogenic medium, which was changed 3 times weekly.

2.5.3. Primary osteoblast culture

Primary mouse osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from 2 day old C57BL/6 mice using a 3-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min) [39]. The first two digests were discarded and the cells resuspended in MEM supplemented with 10% FCS, 2 mM L-glutamine, 1% gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. Cells were cultured for 2–4 days at 37 °C in 5% CO_2 until they reached confluence. They were then cultured in 6-well trays in MEM supplemented with 2 mM β-glycerophosphate and 50 μg/ml ascorbic acid, with half medium changes every 3 days. Exendin-4 (0, 10, 25, 50 and 100 nM) or Liraglutide (0, 10, 100, 500, and 1000 nM) was added to the culture (1 plate/treatment).

Bone nodule formation by osteoblasts was measured after 28 days of culture. Experiments were terminated by fixing cell layers in 4% paraformaldehyde for 10 min; mineralised bone nodules were visualised and quantified unstained. Cell layers were imaged at 800 dpi using a high-resolution flat-bed scanner. Binary images of each individual well were then subjected to automated analysis (ImageJ), using constant "threshold" and "minimum particle" levels, to determine the number and surface area of mineralised bone nodules as previously described [40].

2.5.4. Primary osteoclast culture

Osteoclast formation and activity were studied in an in vitro model in which osteoclasts are derived from the bone marrow of juvenile mice [41]. Bone marrow was flushed from the long bones of two 6 week old female C57BL/6 mice. Marrow cells were centrifuged at 300 *g* and resuspended in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml amphotericin B, containing 10^{-7} M prostaglandin E_2 and 50 ng/ml M-CSF (referred as MEM_1) (R&D Systems Europe Limited, Abingdon, UK). The cell suspension was incubated for 24 h at 37 °C in 5% CO_2 to allow attachment of stromal cells. Non-adherent cells were collected and resuspended at 5×10^6 cells/ml in supplemented MEM (referred as MEM_2) complemented with 10^{-7} M prostaglandin E_2, 150 ng/ml M-CSF and 3 ng/ml RANKL (Receptor activator of nuclear factor kappa-B ligand) (R&D Systems Europe Limited, Abingdon, UK). 106 cells were plated into a 96 well plate containing dentine disks in each well and incubated for 24 h to allow attachment of osteoclast precursors. Dentine disks were then transferred to 6 well trays containing MEM_2. Exendin-4 (Sigma) (0, 10, 25, 50 and 100 nM) or Liraglutide (Bachem) (0, 10, 100, 500, and 1000 nM) was added to the culture in two wells/group (10 disks). Cultures were grown for 8 days with half medium changes every 2–3 days. Culture medium was maintained at pH 7.4 for the first 6 days, then the pH was reduced to 7 by addition of HCl in order to activate osteoclast resorption activity.

Experiments were terminated by washing disks in PBS, followed by fixation in 2.5% glutaraldehyde. Disks were stained for TRAP using a leukocyte acid phosphatase kit (Sigma) according to the manufacturer's instructions. The total number of osteoclasts on each disk was assessed by transmitted light microscopy and the plan surface area of resorption pits was measured using reflective light microscopy and dot-counting morphometry (ImageJ) in blinded fashion [41].

2.6. RT-PCR

Long bones of wild-type C57BL/6 mice were flushed with PBS to remove the bone marrow, then individually powdered with a mortar and pestle under liquid nitrogen. MLO-A5 and IDG-SW3 cells were trypsinised with 0.05% trypsin–EDTA (Life Technologies). Osteoclast RNA was extracted from control conditions as described above at day 2 (OC precursors), day 5 (early OC), day 7 (mature OC) and day 9 (resorbing OC) by scraping the dentine disks. Osteoblast RNA was extracted from control cells as described above at day 7 (proliferating OB), day 14 (differentiating OB) and day 21 (mature-mineralising OB) of culture after addition of 0.05% trypsin–EDTA to the wells.

Total RNA from bones, bone marrow, MLO-A5 cells, IDG-SW3 cells, primary osteoclasts and osteoblasts was extracted using TRIzol® reagent or RNeasy Mini Kit (Qiagen) according to the manufacturers' protocols. Total RNA concentration and purity were estimated by absorbance at 260 and 280 nm, respectively and integrity by visualisation of ribosomal bands after agarose gel electrophoresis.

GLP-1 receptor mRNA was amplified by RT-PCR in 50 μl volumes and the amplification parameters consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The resulting products were visualised by electrophoresis on a 2% agarose gel. The mouse primers used for amplification were 5′-TCCTTCGTGAATGTCA GCTG-3′ and 5′-TGGTGCAGTGCAAGTGTCTG-3′ (designed using Blast).

2.7. Serum quantifications of calcitonin and sclerostin

Quantification of calcitonin in mouse serum was performed using a competitive inhibition enzyme immunoassay, Murine ELISA kit for calcitonin (Uscn Life Science Inc.). Serum levels of sclerostin were detected using a Solid Phase Sandwich ELISA, ®ELISA Mouse/Rat SOST kit (R&D Systems Europe, Ltd., Abingdon, UK) according to the manufacturer's recommendations.

2.8. Statistics

Data are presented as mean ± SD. Multiple comparisons were performed using one-way analysis of variance, using Dunnett's Post-hoc test where appropriate. P values less than 0.05 were considered to be statistically significant. In vitro results are representative of three independent experiments.

3. Results

3.1. Effects of GLP-1 receptor agonist treatment on trabecular bone mass and architecture in ovariectomised mice

We examined the effects of chronic LIR and Ex-4 treatment on bone mass and architecture of OVX mice using micro-CT. There were no significant differences in tibial lengths between the three groups (Table 1). However, LIR and Ex-4 improved bone mass, as both

Table 1
Micro-CT measurements of trabecular and cortical bone architecture in 5-month old ovariectomised mice treated with GLP-1 agonists.

Parameters	Saline	Exenatide (10 μg/kg/day)	Liraglutide (0.3 mg/kg/day)
Length of tibia (mm)	17.8 ± 0.5	17.8 ± 0.3	17.8 ± 0.4
Trabecular architecture			
BV/TV (%)	1.38 ± 0.42	1.89% ± 0.48 *	2.06% ± 0.52 **
Tb.N (number/mm)	0.280 ± 0.97	0.373 ± 0.098 *	0.407 ± 0.085 **
Tb.Pf (factor/mm)	33.31 ± 3.62	29.57 ± 2.42 *	29.04 ± 2.66 **
Tb.Th (mm)	0.0501 ± 0.0048	0.0487 ± 0.0047	0.0502 ± 0.0046
Tb.Sp (mm)	0.676 ± 0.081	0.621 ± 0.076	0.631 ± 0.080
SMI	2.51 ± 0.23	2.33 ± 0.19	2.28 ± 0.080
DA	2.66 ± 0.43	2.86 ± 0.35	3.05 ± 0.29
Cortical architecture			
Tt.Ar (mm^2)	1.238 ± 0.063	1.270 ± 0.079	1.204 ± 0.097
Tt.Pm (mm)	5.34 ± 0.28	5.54 ± 0.35	5.34 ± 0.36
Ct.Ar (mm^2)	0.739 ± 0.054	0.758 ± 0.049	0.732 ± 0.065
Ecc	0.789 ± 0.035	0.806 ± 0.035	0.778 ± 0.042
MMI polar (mm^4)	0.278 ± 0.040	0.301 ± 0.042	0.269 ± 0.050
Ct.Th (mm)	0.201 ± 0.011	0.204 ± 0.011	0.202 ± 0.011

Measurements were assessed by micro-CT in the proximal tibial metaphysis of mice treated with either saline, Liraglutide or Exenatide. BV/TV: bone volume percent, Tb.N: trabecular number, Tb.Pf: trabecular pattern factor, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation, SMI: structure model index, DA: degree of anisotropy, Tt.Ar: tissue area, Tt.Pm: tissue perimeter, Ct.Ar: bone area, Ecc: eccentricity, MMI polar: moment of inertia, Ct.Th: cross-sectional thickness. Mean ± SD of n = 10 mice/group. *, P < 0.05; **, P < 0.01; versus saline.

treatments significantly increased the BV/TV and Tb.N of ovariectomised mice compared to saline-treated controls by 49% and 35% respectively for BV/TV and 33% and 45% respectively for Tb.N (Table 1). LIR and Ex-4 also improved bone structure and connectivity, as shown by the decreases in Tb.Pf by 13% and 11% respectively and SMI by 9% and 7% respectively (only significant with LIR). There was no effect of either LIR or Ex-4 on Tb.Th, Tb.Sp and DA. GLP-1RA had no effect on cortical architecture (Table 1).

3.2. Effects of GLP-1 receptor agonist treatment on in vivo bone turnover

We next aimed to use histomorphometry to corroborate the beneficial effects of GLP-1RA on trabecular bone architecture and to examine bone cellular activities. Bone histomorphometry similarly showed an improvement in trabecular bone mass and architecture as a result of GLP-1RA treatment (Table 2). Both LIR and Ex-4 increased BV/TV and Tb.N in the trabecular bone of ovariectomised mice. Tb.Th was not affected, but Tb.Sp was decreased as a result of treatment with both LIR and Ex-4, when quantified by histomorphometry (Table 2). However, this effect was not observed using Micro-CT (Table 1).

Table 2
Histomorphometric measurements of bone architecture and cellular activities in 5-month old ovariectomised mice treated with GLP-1 agonists.

Parameters	Saline	Exenatide (10 μg/kg/day)	Liraglutide (0.3 mg/kg/day)
BV/TV (%)	3.38 ± 1.69	5.58 ± 2.76 *	6.22 ± 1.90 **
Tb.N (number/mm)	0.98 ± 0.34	1.46 ± 0.50 *	1.47 ± 0.39
Tb.Th (mm)	0.0335 ± 0.0076	0.03376 ± 0.0095	0.0422 ± 0.0061
Tb.Sp (mm)	1.086 ± 0.365	0.710 ± 0.212 *	0.686 ± 0.225 *
TRAP Oc S/BS (μm) % control	100 ± 50	150.6 ± 22.8 *	142.6 ± 31*
TRAP Oc N/BS (1/mm) % control	100 ± 29.7	162 ± 65.6 *	138 ± 30.9
MS/BS (%)	41.3 ± 7.8	43.8 ± 6.6	44.5 ± 5.7
MAR (μm/day)	2.15 ± 0.43	1.96 ± 0.31	1.96 ± 0.32
BFR/BS ($\mu m^3/\mu m^2$/day)	0.890 ± 0.231	0.846 ± 0.216	0.875 ± 0.194
Adipocyte number (number/mm^2)	93 ± 32	92 ± 44	103 ± 43

Cellular parameters were measured by bone histomorphometry on sections of the trabecular region of mouse tibia from mice treated with saline, Liraglutide (0.3 mg/kg/day) or Exenatide (10 μg/kg/day). BV/TV: bone volume percent, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation, TRAP Oc·S/BS: osteoclastic surfaces per millimetre of trabecular bone surface, TRAP Oc·N/BS: number of osteoclast per millimetre of trabecular bone surface, MS/BS: alizarin red-labelled surfaces per bone surfaces, MAR: mineral apposition rate, BFR: bone formation rate. Mean ± SD of n = 10 mice/group, *P < 0.05 **, P < 0.01 versus saline.

Ex-4 and LIR significantly increased the percentage of TRAP-positive surfaces (osteoclast surfaces) but only EX-4 enhanced the number of osteoclasts compared to control mice (Table 2). Analysis of bone formation activity showed that mineralising surfaces and MAR were not affected by either Ex-4 or LIR treatment (Table 2). Bone formation rate was also not changed by GLP-1 agonist treatment (Table 2). In addition, Ex-4 and LIR had no effect on adipocyte number in the trabecular bone (Table 2).

3.3. Effects of GLP-1 receptor agonists on serum calcitonin and sclerostin levels

Serum calcitonin concentration was increased by 177% in the Ex-4-treated mice versus the control mice. However, calcitonin levels in LIR-treated mice were not significantly elevated (Fig. 1A).

Serum sclerostin was 23% lower in the Ex-4-treated mice than in control mice. However, as with calcitonin, no difference in sclerostin levels was observed between LIR-treated mice and control mice (Fig. 1B).

3.4. Expression of GLP-1 receptor in bone tissue and bone cells

To determine whether the observed effects of GLP-1RA treatment might be mediated through a direct skeletal mechanism, we next determined whether the pancreatic-type GLP-1R is expressed in mouse bone and bone marrow, primary osteoblasts and osteoclasts and osteocyte-like MLO-A5 and IDG-SW3 cells. As shown in Fig. 2A, GLP-1R mRNA expression was detected in mouse bone and bone marrow. Although no expression of the GLP-1R mRNA was detected in the MLO-A5 osteocytic cell line, it was detected in the IDG-SW3 osteocytic cell line. Fig. 2 (C, D) illustrates the presence of GLP-1R mRNA in primary osteoblasts and osteoclasts at different stages of differentiation. In order to further validate these results, we examined the distribution of GLP-1R in mouse bone using immunohistochemistry using muscle as a control of the specificity of GLP-1R antibody (Fig. 3). GLP-1R was expressed in the periosteum (Fig. 3B), bone marrow cells (Fig. 3C) and in some osteocytes, but not all (Fig. 3D). Immunohistochemistry and TRAP staining of consecutive trabecular bone sections confirmed that GLP-1R was also expressed in osteoclasts (Fig. 3E).

3.5. Effects of GLP-1 receptor agonists on osteoclast number and activity in vitro

As GLP-1R is expressed in osteoclasts, we next determined whether GLP-1RA could influence bone resorption in vitro. To determine whether GLP-1RA can directly affect osteoclastic formation and activity, osteoclast progenitors from mouse bone marrow were cultured with either Ex-4 (10 nM to 100 nM) or LIR (25 nM to 1000 nM) (Fig. 4). Osteoclast number was increased in a dose dependent manner for Ex-4 and was enhanced at 1000 nM for LIR. However, addition of Ex-4 (≥50 nM) to mature osteoclasts decreased bone resorption per osteoclast by up to 20%, while LIR (1000 nM) decreased bone resorption per osteoclast by up to 13%, showing that despite an increase in the number of osteoclasts, the overall resorption is decreased.

3.6. Effect of GLP-1 receptor agonists on bone formation in vitro

To determine whether GLP-1RA can directly affect in vitro bone formation, mouse osteoblast progenitors were cultured in osteogenic medium with either Ex-4 (10 nM to 100 nM) or LIR (25 nM to 1000 nM) (Fig. 5). As previously published, abundant mineralised bone nodules

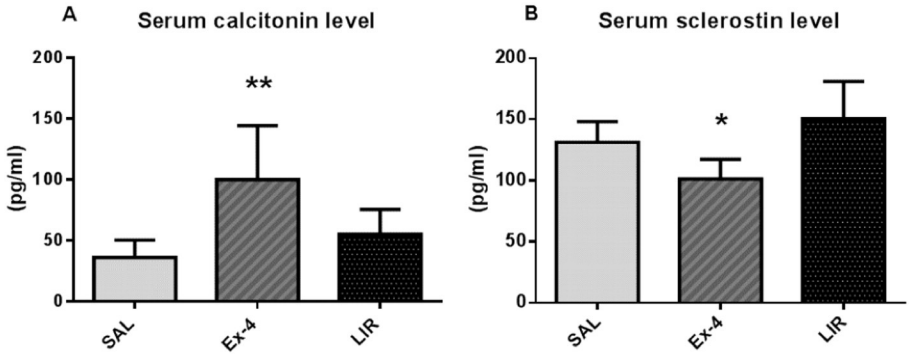

Fig. 1. Effect of GLP-1RA on serum calcitonin and sclerostin levels in 5-month-old ovariectomised mice. (A) Calcitonin levels were assessed by competitive ELISA and (B) sclerostin levels were assessed by sandwich ELISA in serum from 5-month-old ovariectomised mice treated with saline, Liraglutide (0.3 mg/kg/day) or Exenatide (10 μg/kg/day). Bars represent mean ± SD of n = 5 mice/group. *, P < 0.05 versus control.

with characteristic trabecular features formed after 4 weeks of culture [39]. However, neither treatment had an effect on the area of bone nodule formation compared to control.

4. Discussion

This is an extensive study that has examined the long-term effects of two different GLP-1RA on bone mass, architecture and bone cellular activities in situ, coupled with assessment of the skeletal expression of GLP-1R and the effects of these two GLP-1RA on bone resorption and formation in vitro. We demonstrate that a 4 week treatment with two GLP-1RA improves trabecular bone mass and architecture in ovariectomised mice, despite no effect of Ex-4 and LIR on bone formation in vivo and an increase in osteoclast number and surfaces with Ex-4. We also show that GLP-1R is expressed in bone cells, implying that GLP-1RA could exert direct effects on the bone.

The demonstration that GLP-1RA improve trabecular bone mass in ovariectomised mice corroborates the results of previous studies that have reported osteogenic effects of GLP-1RA in normal, IR, T2DM and ovariectomised rodent models [23–28]. In most of these studies, GLP-1RA treatment was administered for 3 days by continuous infusion while we investigated the longer term skeletal effect of LIR and Ex-4 in OVX mice, having administered these drugs daily for 4 weeks. We examined bone architecture together with bone cellular activity in situ. Both drugs increased trabecular bone volume in tibiae, mostly manifested by an increase of the number of trabeculae rather than modifications in their thickness. Silva and Gibson [42] have shown that it is more important to maintain trabecular number than thickness to preserve bone mass, indicating that GLP-1RA which restore the number of trabeculae in the bone could be advantageous in individuals predisposed towards osteoporosis. Our results also indicate an improvement in trabecular architecture with GLP-1RA treatment and a normalising effect upon the OVX-impaired connectivity and anisotropy factors (Tb.Pf and SMI), effects that have also been observed in short term studies with GLP-1RA in T2DM and IR models [24,28]. These positive effects of GLP-1RA on bone mass and architecture were confirmed in histology sections using bone histomorphometry. Since we did not have a sham group, we cannot however know whether GLP-1 agonists improved the trabecular bone loss induced by OVX to the level of control. In contrast, GLP-1RA had no effect on the cortical bone, also in agreement with most previous studies [27,28], although one recent paper did report a beneficial effect of LIR on cortical thickness and area in T2DM rats [27]. This variation could be explained by the fact that bone turnover is different in a mouse model of T2DM and after OVX and that LIR was administered to growing 2 week-old male diabetic mice in the previous study while we used female adult mice in our study. Ma et al. [28] found a greater effect of Ex-4 on the trabecular bone than ours at a dose of 3 μg/kg/day, but the rats received the treatment for 16 weeks.

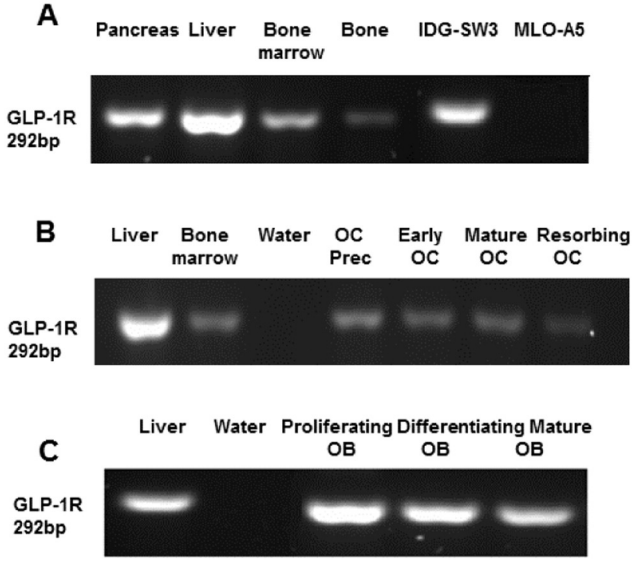

Fig. 2. Expression of GLP-1 receptor mRNA in mouse tissues and bone cells using RT-PCR. (A) The GLP-1 receptor mRNA is expressed in bone marrow, bone, in the late osteocytic cell line IDG-SW3 but not in the pre-osteocytic cell line MLO-A5. Liver and pancreas were used as positive controls. (B) Expression of the GLP-1 receptor mRNA in primary osteoclasts (OC) derived from mouse bone marrow at various stages of differentiation (OC precursors, early OC, mature OC and resorbing OC). (C) Expression of the GLP-1 receptor mRNA in primary osteoblasts (OB) at different stages of maturation (proliferating OB, differentiating OB and mature mineralising OB).

No previous report has compared the skeletal effects of LIR and Ex-4. Our data indicate that LIR and Ex-4 have similar effects on bone architecture and cellular activities, although they differentially affect serum calcitonin and sclerostin, suggesting possible different mechanisms of action in the bone. It is conceivable that some disparity between these drugs might have been created by the particular doses chosen. The half-life of Ex-4 is 2.4 h while LIR has a half-life of 13 h, implying that Ex-4 would need to be given in more frequent doses to build up and maintain a high enough concentration in the blood to be therapeutically effective [43]. LIR treatment is formulated as once daily injection in contrast to Ex-4, which is administered twice daily [44]. However, in our study we administrated both treatments once daily and this may have contributed to the less pronounced effect of Ex-4 on bone, although the doses for both drugs were chosen because they had osteogenic effects in previous studies [23–26,28]. Clinical studies have shown that LIR is more efficacious than Ex-4 in patients with T2DM, as it induces a significant greater reduction in HbA1c [43].

Little is known about GLP-1's putative role as a modulator of bone turnover. Mice with homozygous deletion of GLP-1 receptor develop cortical osteopenia and bone fragility, as well as increased osteoclastic bone resorption, which might be due to a reduction in thyroid calcitonin

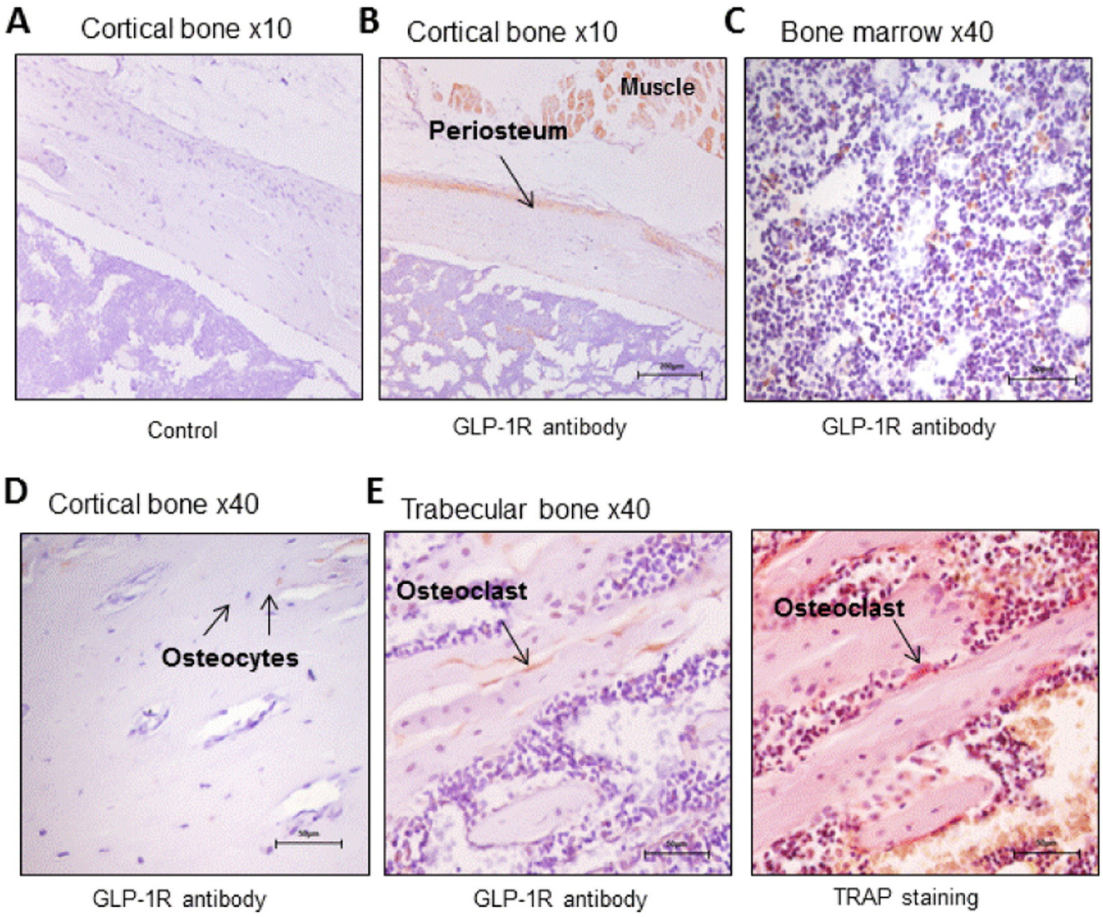

Fig. 3. Expression of the GLP-1 receptor in mouse femur by immunohistochemistry. Immunostaining of mouse femur with GLP-1R antibody and counterstain with haematoxylin. (A) Cortical bone stained with rabbit IgG as a negative control ×10. (B) Cortical bone staining ×10, (C) bone marrow ×40, (D) osteocytes in cortical bone ×40, (E) trabecular bone ×40; consecutive sections immunostained with GLP-1R antibody and TRAP-stained respectively to visualise osteoclasts.

secretion [20]. Although the mechanisms by which GLP-1 regulates bone turnover are unknown, in vitro studies suggest that GLP-1 might directly affect osteoblastic cells via a specific GLP-1 receptor, different or similar to the one expressed in the pancreas. There are indeed divergences regarding the identity of the GLP-1R in bone. Murine MC3T3 osteoblastic cells were shown to express a GLP-1 receptor different from the cloned GLP-1 receptor in the pancreas [29], while human osteoblastic cell lines and osteocytes in vivo and in vitro express the GLP-1R mRNA [31], which was not detected in primary bone cells cultured on plastic [26]. Using RT-PCR, we demonstrate that GLP-1R mRNA is expressed in the bone, bone marrow cells and mouse osteoblasts isolated from calvaria and cultured for 28 days. These mature osteoblasts express all the markers of differentiated osteoblasts and form bone nodules in vitro [39]. We also found expression of GLP-1R in osteoclasts using an in vitro bone resorption model where osteoclasts are cultured on dentin disks to stimulate their differentiation and activity, which is more comparable to the in vivo situation [41]. Our results show no expression of GLP1-R in the early osteocytic cell line MLO-A5, while it was expressed in the late osteocytic cell line IDG-SW3. These data were confirmed in vivo using immunohistochemistry and we found that GLP-1R was specifically expressed in the periosteum, bone marrow, osteoclasts and in some osteocytes but not all, suggesting that expression may be influenced by osteoblastic differentiation status. Only one previous study assayed GLP-1R expression in bone in situ, which similarly identified the receptor in some osteocytes [26].

The presence of GLP-1R in bone cells in vitro and in situ implies that GLP-1RA could have a direct effect on bone cells. Since GLP-1RA improved trabecular bone architecture, we examined bone resorption and formation activities in vivo in trabecular bone using bone histomorphometry and in vitro using bone resorption and formation assays. We showed no direct effect of GLP-1RA on bone formation and mineralisation rates in vivo and no change in mineralised bone nodule formation in vitro. In contrast, GLP-1RA significantly increased the number of bone resorption surfaces in vivo. As the measurement of osteoclast surfaces with histomorphometry does not give a direct indication of whether osteoclastic resorptive activity is affected, we investigated the effects of GLP-1RA on osteoclastogenesis in vitro and our data show that both LIR and EX-4 significantly increase osteoclast number, while decreasing the area resorbed per osteoclast, suggesting that GLP-1RA stimulate osteoclastic differentiation but impair their resorptive activity. Our results contrast those from Ma et al. [28], that showed enhanced bone formation and decreased numbers of osteoclasts per millimetre of trabecular bone surface after 16 weeks of administration of Ex-4 to OVX rats. The reason for this discrepancy is unclear, but it may be possible that the bone loss induced by OVX in our experiment was too severe to measure any significant changes in bone formation, as the trabecular bone volume percent was very low (1–2%). Both mice and rats experience rapid bone loss following ovariectomy, but we have previously observed that the trabecular bone volume in C57Bl/6NCrl mice is relatively low compared to that of rats. The increase in bone resorption surfaces as a result of GLP-1 agonist treatment is surprising, as it is known that incretins inhibit bone resorption after the ingestion of fat or protein [17,18] and that daily administration of GLP-2 decreases bone resorption markers [45]. It is difficult to understand why bone resorption is increased after GLP-1RA treatment in our study. We did not measure the OPG/RANKL ratio but our in vitro data indicate that GLP-1RA may favour osteoclast formation from precursors. It is also challenging to bring together the direct effects of GLP-1RA on bone cellular activities with the increase in trabecular

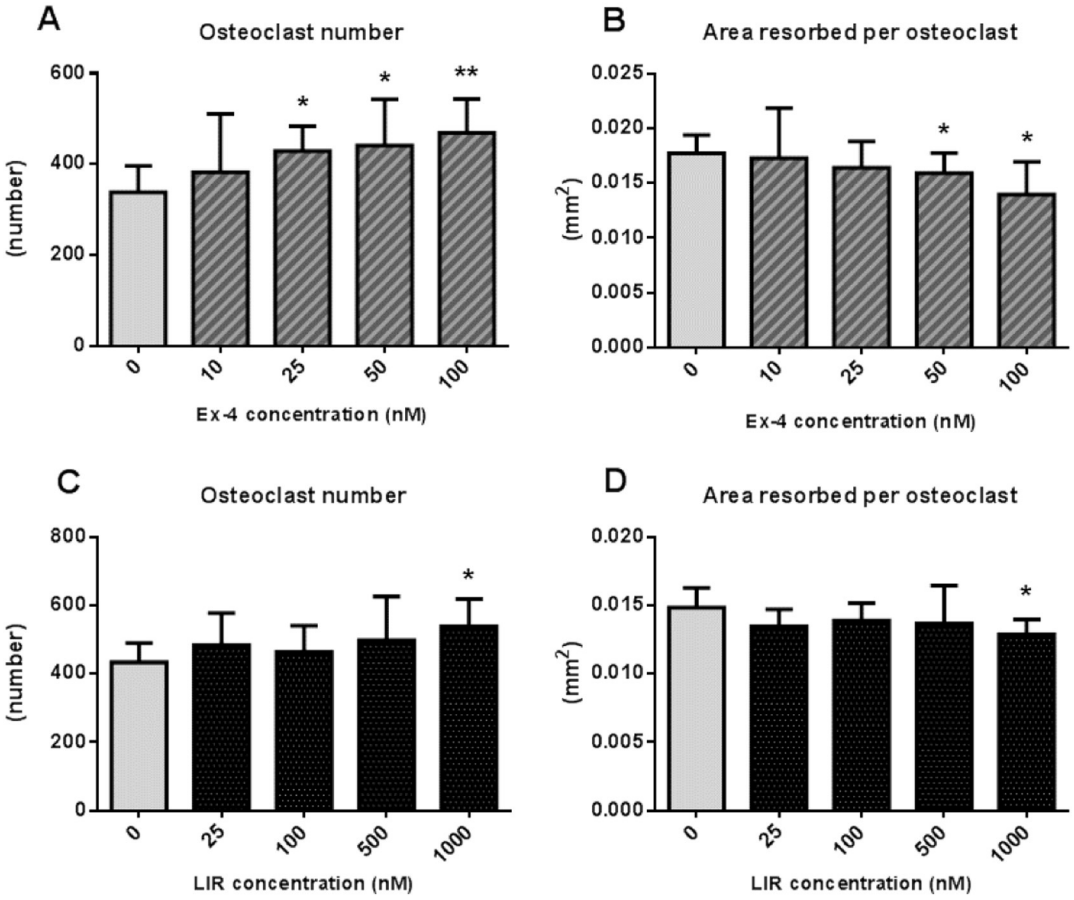

Fig. 4. Effects of GLP-1RA on osteoclastic differentiation and bone resorption in vitro. Primary osteoclasts were isolated from mouse bone marrow and cultured on dentine disks. Either Exenatide or Liraglutide was added to the medium at various concentrations. Osteoclasts were stained for TRAP activity and viewed by transmitted light. (A) Number of osteoclasts per dentine disk in cultures treated with Exenatide, (B) resorption area per dentine disk in cultures treated with Exenatide, (C) number of osteoclasts per dentine disk in cultures treated with Liraglutide, (D) resorption area in cultures treated with Liraglutide. Mean ± SD of 10 disks/group. **, $P < 0.01$; *, $P < 0.05$ versus control.

bone volume that we observed. The inhibition of osteoclast activity induced by Ex-4 and LIR may partly explain the increase in trabecular bone mass, although this is probably not the main mechanism of action of GLP-1RA in the bone, as the reduction in bone resorption is very modest. Although GLP-1RA do not directly affect osteoblast activity, we cannot exclude however that bone formation duration is overall increased with GLP-1RA indicating that the active formation period of a basic structural unit of the bone is augmented. This can be determined by the average volume of bone matrix made by each team of osteoblasts and is revealed by the measurement of the wall thickness, which is rather difficult to measure in mice [46]. Differentiation of bone marrow mesenchymal stem cells (BMMSCs) into osteoblasts or adipocytes is crucial for bone remodelling and our immunohistochemistry data show that GLP-1R is expressed both in bone marrow and in the periosteum, which contains

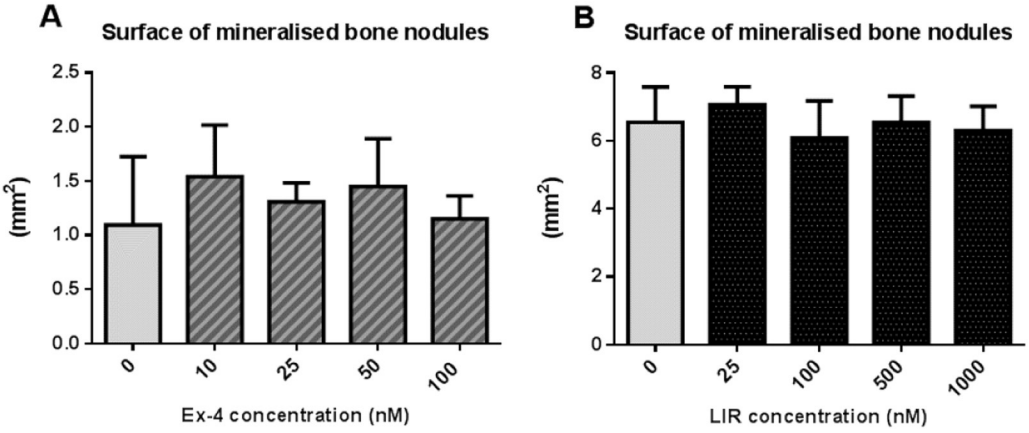

Fig. 5. Effects of GLP-1RA on bone nodule formation in vitro. Either Exenatide or Liraglutide was added to the 28-day primary culture of mouse osteoblasts isolated from calvarial bone and cultured in osteogenic medium at increasing concentrations. (A) Area of bone formation representing the mineralised bone nodule observed with reflected light scans of unstained cell wells by osteoblasts cultured with Exenatide, (B) area of bone formation by osteoblasts cultured with Liraglutide. Mean ± SD of 6 wells/group. *, $P < 0.05$ versus control.

many osteoprogenitor cells. We cannot exclude that BMMSCs are also a key target of GLP-1RA, although we did not observe any effect of these drugs on the number of adipocytes in the bone marrow in situ. Previous in vitro studies have shown a reduction in adipocyte differentiation from BMMSCs when cultured with Ex-4 [28].

It is also possible that the main skeletal effects of GLP-1RA are not directly on bone cells. It has been shown that GLP-1R is also expressed in C-cells of the thyroid gland and exerts, when activated by GLP-1 or its stable analogues, a stimulating effect on calcitonin secretion in rodents [47,48], a potent inhibitor of bone resorption. As proposed by Yamada et al. [20], GLP-1RA effects on bone might be indirect rather than direct, acting mainly by targeting calcitonin secretion from the thyroid to modulate bone turnover. Our data indicate that Ex-4 increases serum calcitonin while we found no effect of LIR on plasma calcitonin, in contrast to previous studies that have reported increased plasma calcitonin after both Ex-4 and LIR treatment [47,48]. Nevertheless, our data are not completely consistent with a calcitonin-mediated mechanism, since we observed an increase in resorption surfaces after GLP-1RA treatment, whereas other studies have reported that calcitonin decreased resorption surfaces [49]. However, calcitonin principally suppresses osteoclast activity, which is not always reflected in altered osteoclast surfaces.

Sclerostin, the protein product of the SOST gene, can bind to bone morphogenetic proteins (BMP) and inhibit canonical Wnt/β-catenin signalling, which is essential for bone formation [50]. Interestingly, sclerostin levels are increased in diabetes and can be regulated by GLP-1RA [4,26]. We show here that Ex-4 treatment reduces serum sclerostin in mice. These findings are in agreement with Kim et al. [26] and suggest an alternative mechanism that could contribute to the increased trabecular bone mass in mice treated with Ex-4. However, LIR had no effect on sclerostin production. The effect of LIR on sclerostin production has not been investigated by others and it is unclear yet why there is this major difference between LIR and EX-4. Further studies on the mechanisms of actions of these drugs are required.

5. Conclusion

Taken together, our results suggest that GLP-1RA improve trabecular bone mass and architecture in ovariectomised mice, although their mechanism of action is still unclear. Our findings may guide selection of therapeutic strategies to prevent and improve the low bone mass and deterioration of bone tissue associated with aged postmenopausal osteoporosis. Considering the impaired bone structure and elevated risk of fractures in diabetic patients, the use of GLP-1RA as an antidiabetic therapy may have additional beneficial effects on the skeleton, avoiding the occurrence of additional diabetic complications. More studies are therefore required to examine the long-term potential beneficial effects of GLP-1 therapy in diabetic patients that are at risk of concurrent osteoporosis.

Disclosure statement

The authors have nothing to disclose.

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