GLP-1 and exendin-4 can reverse hyperlipidic-related osteopenia

Bernardo Nuche-Berenguer¹, Daniel Lozano², Irene Gutiérrez-Rojas¹, Paola Moreno¹,
María L Mariñoso³, Pedro Esbrit^{2,*} and María L Villanueva-Peñacarrillo^{1,*}

¹Department of Metabolism, Nutrition and Hormones and ²Bone and Mineral Metabolism Laboratory, Instituto de Investigación Sanitaria (IIS)-Fundación Jiménez Díaz, Avda Reyes Católicos 2, 28040 Madrid, Spain

³Department of Pathology, Hospital del Mar, 08003 Barcelona, Spain

(Correspondence should be addressed to M L Villanueva-Peñacarrillo; Email: mlvillanueva@fjd.es)

*(P Esbrit and M L Villanueva-Peñacarrillo contributed equally to this work)

Abstract

Increased fat mass contributes to bone deterioration. Glucagon-like peptide 1 (GLP-1) and its related peptide exendin 1–39 amide (Ex-4), two lipid-lowering peptides, exert osteogenic effects in diabetic states. We examined the actions of 3-day administration of GLP-1 or Ex-4 on bone remodeling markers and on bone mass and structure in hyperlipidic (HL) and hypercaloric rats. Wistar rats on a hyperlipidemic diet for 35 days were subcutaneously administered GLP-1 (0.86 nmol/kg per h), Ex-4 (0.1 nmol/kg per h), or saline (control) by continuous infusion for 3 days. After killing, tibiae were removed for total RNA and protein isolation, as well as femurs and L1–L4 vertebrae for bone mass and quality assessment. Body weight and plasma insulin were unaltered in HL rats, which showed osteopenia (by dual-energy X-ray absorptiometry), associated

with hyperglycemia, hypertriglyceridemia, and hypercholesterolemia. GLP-1 or Ex-4 administration decreased the levels of glucose, triglycerides, and total cholesterol in plasma but increased osteocalcin (OC) gene expression and the osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) ratio – at the expense of an augmented OPG – above corresponding control values in the tibia. Each tested peptide similarly reversed the decreased femoral and vertebral bone mass in these rats, whereas the deteriorated trabecular structure in the vertebrae improved associated with normalization of bone remodeling. These findings demonstrate that GLP-1 and Ex-4 are similarly efficient in reversing the bone alterations in this HL rat model, which has proven to be useful for studying the fat–bone relationships.

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Introduction

Obesity is closely related to hypertension, insulin resistance, diabetes mellitus, and dyslipidemia, which significantly increase the risk of cardiovascular disease (see Hummasti & Hotamisligil (2010) for review). Although obesity has been traditionally considered protective for the skeleton, increasing evidence suggests otherwise. It now seems to be clear that this apparent protection is a consequence of the mechanical loading imposed by high body weight in this condition (Rosen & Bouxsein 2006, Pereira *et al.* 2007). In fact, hypercholesterolemia appears to contribute to the pathogenesis of osteoporosis in postmenopausal women, which might justify the use of cholesterol-lowering statins as putative therapies in this situation (Herrington & Potvin Klein 2001, Tanko *et al.* 2003, Majima *et al.* 2007). In the Zucker diabetic fatty rat model, osteopenia was prevented by the administration of insulin-sensitizing agents lowering hyperlipidemia and hyperglycemia (Shibata *et al.* 2000). In addition, a correlation between obesity and fractures – likely a

consequence of an altered bone mass and/or bone fragility – has recently been reported in postmenopausal women (De Laet *et al.* 2005, Premaor *et al.* 2010). Fat and bone metabolism are functionally related through complex neuroendocrine pathways (Reid 2010) and also by the fact that adipocytes and osteoblasts share a common progenitor from bone marrow stromal cells (Rodriguez *et al.* 1999). In this respect, the negative bony effect of fat is particularly evident in the bone marrow milieu of aging and osteoporotic subjects, which shows high adiposity and decreased osteoblastic differentiation and bone formation (Rodriguez *et al.* 1999, Verma *et al.* 2002, Rosen & Klibanski 2009). In addition, hyperlipidemia has been shown to promote osteoclastogenesis and bone resorption (Tintut *et al.* 2004). Hence, the majority of studies point to increased fat mass as an important factor contributing to poor bone quality in obese subjects (Zhao *et al.* 2008, Buizert *et al.* 2009).

It has been reported that the anti-diabetic peptides glucagon-like peptide 1 (GLP-1) and exendin 1–39 amide (Ex-4) show beneficial effects in reducing cholesterol and

triglycerides in diabetic subjects (Klonoff *et al.* 2008, Rizzo *et al.* 2009, Horton *et al.* 2010). GLP-1, an incretin with insulinotropic and insulin-independent anti-diabetic properties (Creutzfeldt 2001), exerts insulin-3-like effects upon glucose metabolism in the liver, the skeletal muscle, and fat, apparently through a specific receptor different from that cloned in the pancreas (Valverde *et al.* 1994, Delgado *et al.* 1995, Villanueva-Peñacarrillo *et al.* 1995, Yang *et al.* 1998, Villanueva-Peñacarrillo *et al.* 2001b, Sancho *et al.* 2005, Arnés *et al.* 2008). In adipose tissue, GLP-1 has opposite actions upon lipid metabolism – lipogenic and lipolytic – which are mediated by cAMP and inositolphosphoglycan (IPG) signaling pathways respectively (Ruiz-Grande *et al.* 1992, Márquez *et al.* 1998, Villanueva-Peñacarrillo *et al.* 2001a). Ex-4 is a natural occurring non-mammalian peptide that shows partial homology to GLP-1 and shares with this incretin several glucose and lipid regulatory effects in both normal and diabetic states (Eng *et al.* 1992). However, Ex-4 has a much longer circulating half-life than GLP-1; it is resistant to degradation by dipeptidyl peptidase IV, which makes it more suitable as an anti-diabetic agent (Nielsen *et al.* 2004). Similar to GLP-1, Ex-4 also lowers gastric emptying and has anorectic properties (Willms *et al.* 1996, Szayna *et al.* 2000, Talsania *et al.* 2005).

Recently, we demonstrated that both GLP-1 and Ex-4 exert osteogenic effects in rats with either type 2 diabetes – by administering a single dose of streptozotocin on the day of birth – or fructose-induced insulin resistance, as shown by alterations in various osteoblastic genes and bone structural parameters (assessed by micro-computed tomography (μ CT)) in the appendicular skeleton (Nuche-Berenguer *et al.* 2009, 2010a). These studies suggest that the osteogenic action of these peptides might be in part a consequence of osteoclast inhibition through changes in the osteoprotegerin (OPG)/receptor activator of NF- κ B ligand (RANKL) ratio in osteoblasts. Moreover, it has recently been reported that GLP-1 directly interacts with specific receptors that are independent of its cAMP-linked receptor in the pancreas but dependent on IPG in an osteoblastic cell line (Nuche-Berenguer *et al.* 2010b). Collectively, the recently reported bony effects of GLP-1 and Ex-4, together with their known lipid-lowering action, make them candidates for improving bone health in hyperlipidemia-related conditions.

In this study, we aimed to evaluate the relative efficacy of GLP-1 and Ex-4 as osteogenic agents – by assessing bone mass and several bone turnover markers as well as bone histomorphometric parameters – in a previously characterized hyperlipidic and hypercaloric rat model (Prats *et al.* 1989).

Materials and Methods

Reagents

Human GLP-1 (7–36) amide and Ex-4 (Bachem AG, Bubendorf, Switzerland); porcine insulin (Novo Biolabs,

Bagsvaerd, Denmark); osmotic pumps (Alzet 1003D; Alza, Palo Alto, CA, USA); Meritene mineral and vitamin complex (Nestlé Nutrition, Barcelona, Spain); demeclocycline, TRI Reagent for RNA isolation, and anti- α -tubulin antibody (Sigma-Aldrich); Taqman Universal PCR master mix and high-capacity cDNA reverse transcription kit, probes and primers for rat osteocalcin (OC) (Rn00566386_g1), OPG (Rn00563499_m1), RANKL (Rn00569289_m1), and eukaryotic 18S (4319413E) (Applied Biosystems, Foster City, CA, USA); HRP-conjugated donkey anti-rabbit and anti-mouse IgG, rainbow markers, ECL-western blotting kit, and Hyperfilm ECL (Amersham Pharmacia Biotech); rabbit polyclonal OPG and RANKL antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); transfer semidry system (Trans-blot SD semidry transfer cell, Bio-Rad); and methylmethacrylate (Merck Pharma Química, S.A.). All other commonly used chemicals were from Sigma-Aldrich or Merck.

Animals

We used 6-week-old male Wistar rats kept on a standard pellet diet (Panlab, Barcelona, Spain) and tap water *ad libitum*. Animals were divided into two dietary sets for 35 days: one group of normal rats (N; $n=6$) was fed a standard chow (8% calories as fat) and another group (hyperlipidemic (HL); $n=28$) was fed a ‘cafeteria diet’ consisting of standard chow combined with a daily intake of cookies, liver paste, bacon, and whole milk supplemented with sucrose (333 g/l) and 10 g/l of a mineral and vitamin complex (65% of calories derived from lipids). All the food items were weighed daily and offered in excess to rats. This diet protocol has been shown to provide a high-energy and high-fat intake (Prats *et al.* 1989). A control of daily caloric intake for the 35-day period of the study was done in 12 HL rats, by measuring the leftovers after consumption of each item in the diet, and considering their caloric density provided by the respective manufacturer.

Experimental design

To avoid possible changes associated with aging, all rats in the study were 11 weeks old by the time of each treatment as follows. We subjected two separate groups of HL rats ($n=8$ each) to a 3-day treatment (Nuche-berenguer *et al.* 2009, 2010a) with either GLP-1 (0.86 nmol/kg per h) or Ex-4 (0.1 nmol/kg per h), dissolved in saline, by continuous infusion through a subcutaneously implanted osmotic pump (Alzet 1003D; Alza). Another group of HL rats ($n=12$) were treated only with saline solution in parallel with each peptide group of animals and used as treatment controls. Some of the rats ($n=5$) from each experimental HL group were intraperitoneally injected with demeclocycline (20 mg/kg) at days 13 and 4 before killing. Just before (basal) and at the end of the 3-day peptide (or saline) treatments, blood samples were collected from the rat tail for measuring plasma glucose,

insulin, cholesterol, triglycerides, and calcium and creatinine. Afterward, the animals were stunned and killed by a sharp blow to the head; the tibiae were quick-frozen and stored at -70°C until total RNA and protein isolation; femurs and L1–L4 vertebrae were also collected, stripped of soft tissue, and kept in 70% ethanol for the analysis of bone mineral density (BMD), bone mineral content (BMC), and/or bone histomorphometry. Animal housing and protocols were approved by the Animal Use Committee of the Instituto de Investigación Sanitaria (IIS)-Fundación Jiménez Díaz.

Plasma measurements

Glucose was determined by the glucose oxidase method (Glucose analyzer 2; Beckman, Galway, Ireland); insulin was measured by RIA (Herbert *et al.* 1965, Valverde *et al.* 1988), using rat insulin (Linco, St Charles, MO, USA) as standard; cholesterol and triglycerides were assayed by an automatic analyzer (Advia 1200, Siemens, Bayer).

Isolation of total bone RNA and real-time PCR

The quick-frozen rat tibiae were individually powdered with a cold steel mortar and pestle and then mixed with TRI Reagent for total RNA extraction, following the manufacturer's instructions. Total RNA concentration and purity were estimated by absorbance at 260 and 280 nm, respectively, in an aliquot volume of each sample. cDNA was synthesized from 4 μg total bone RNA using avian myeloblastosis virus reverse transcriptase (Promega) with random hexamer primers. PCR amplification was carried out in a StepOne system (Applied Biosystems), in triplicate for each sample, in a total volume of 20 μl containing 400 ng of cDNA, 900 nM of each primer, 500 nM of the respective probe, and 6 μl of TaqMan Universal PCR Master Mix, as described previously (Nuche-Berenguer *et al.* 2009, 2010a). For each rat, the gene expression was normalized with that of the housekeeping gene *18S* and expressed as $2^{-\Delta\Delta C_T}$.

Immunoblotting

Total proteins from the rat tibiae were isolated, following a previously described protocol (Moreno-Rubio *et al.* 2010). Protein content in bone tissue extracts was determined by the Bradford's method (Pierce, Rockford, IL, USA), using BSA as standard. Equal amounts of proteins (30 μg for OPG and 60 μg for RANKL) were separated on 8% polyacrylamide-SDS gels under reducing conditions. After electrophoresis, the samples were transferred onto nitrocellulose membranes in a Trans-blot SD semidry transfer cell (Bio-Rad). For immunodetection, OPG and RANKL antibodies and anti- α -tubulin were used as primary antibodies and a HRP-conjugated donkey anti-rabbit or anti-mouse IgG as secondary antibodies, subsequently followed by enhanced

chemiluminescence and quantification by densitometry scanning of the autoradiogram, according to a standard protocol (Redondo *et al.* 2003). The densitometry values of the bands corresponding to OPG and RANKL protein were normalized with respect to that of α -tubulin.

Dual-energy X-ray absorptiometry and bone histomorphometry

BMD and BMC were determined in the total femur and lumbar spine (L1–L4) of each rat by using a PIXImus X-ray densitometer (GE Lunar Corp., Madison, WI, USA). The vertebral bone specimens were dehydrated in graded ethanols and embedded in methylmethacrylate. Sagittal longitudinal sections (7 μm thick) of the vertebrae were obtained with a rotation microtome for hard materials (Leica RM2255, Leica Microsystems, Nussloch, Germany) and were stained with von Kossa and Goldner's trichrome for the analysis of static parameters (Campodarve *et al.* 1993). From each biopsy, two sections were kept unstained for subsequent fluorescence microscopy examination (analysis of dynamic parameters). Histomorphometric measurements were performed using a light microscope with reticule-mounted eyepiece grid, and variables were calculated following the American Society for Bone and Mineral Research recommendations (Parfitt *et al.* 1987). The mineralized surface was calculated as: (double label surface + 0.5 single labeled surface)/bone surface (MS/BS). Mineral apposition rate (MAR) and bone formation rate (BFR) were calculated as the separation between two labels/9 days and (MAR $\times$ MS/BS) respectively.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M., together with the number of observations. The statistical significance ($P < 0.05$) of the increments was assessed either by one-way ANOVA followed by the least significant differences test for *post-hoc* multiple comparisons or Student's *t*-test when appropriate, using the Statistical Package for the Social Science (SPSS) software (SPSS Inc., Chicago, IL, USA).

Table 1 Body weight and plasma measurements in normal rats (N), and in hyperlipidemic rats (HL) before (basal) and after 3-day treatment with GLP-1 or Ex-4. Data are mean $\pm$ S.E.M. ($n=8$)

	N	HL		
	Basal	Basal	GLP-1	Ex-4
Glucose (mg/dl)	102 $\pm$ 2	115 $\pm$ 3*	107 $\pm$ 4	100 $\pm$ 3 [†]
Insulin (ng/ml)	1.6 $\pm$ 0.3	1.3 $\pm$ 0.2	1.0 $\pm$ 0.1	1.3 $\pm$ 0.4
Total cholesterol (mg/dl)	67 $\pm$ 4	94 $\pm$ 5*	81 $\pm$ 2 [†]	82 $\pm$ 3 [†]
Triglycerides (mg/dl)	105 $\pm$ 5	147 $\pm$ 17*	83 $\pm$ 7 [†]	95 $\pm$ 9 [†]
Weight (g)	315 $\pm$ 7	337 $\pm$ 9	349 $\pm$ 10	322 $\pm$ 8

* $P < 0.05$ versus N basal value; [†] $P < 0.05$ versus respective HL basal value.

Figure 1 OPG and RANKL mRNA and protein expression in the tibia of normal (N) and hyperlipidemic (HL) rats, untreated (grey) or treated with GLP-1 (black) or Ex-4 (white) as described in the text. OPG and RANKL mRNA levels (assessed by real-time PCR) were calculated as copy numbers by using the Ct value relative to that in N rats, after normalization against 18S rRNA ($2^{-\Delta\Delta C_t}$) value. Protein levels (evaluated by western blot), corresponding to densitometry values of bands normalized to that of α tubulin, were expressed as percent increment of those of the N group. Representative autoradiogram images, which were grouped from different parts of the same gel, are shown in each case (western blot). Protein loading was similar in each well, as assessed by Ponceau S staining (not shown). Data are mean $\pm$ S.E.M. corresponding to 8–12 rats. * $P < 0.05$ or lower versus N; # $P < 0.05$ or lower versus HL.

Results

Plasma measurements

As expected (Prats *et al.* 1989), HL rats showed hyperglycemia, hypertriglyceridemia, and hypercholesterolemia compared with those in the N group (overall mean increase of these parameters: $37 \pm 5\%$ N-rats, $P < 0.02$). A 3-day treatment of HL rats with either GLP-1 or Ex-4 induced a significant decrease in total cholesterol and plasma triglycerides; glycemia was also reduced, but only significantly for Ex-4 (Table 1). No significant differences were detected in body weight or plasma insulin between N and HL rats throughout the study. Plasma calcium and creatinine were also similar in HL and N rats and unchanged by either peptide treatment (not shown), as expected from our previous findings (Nuche-Berenguer *et al.* 2009, 2010a).

Expression of bone markers

In HL rats (Fig. 1), OC mRNA levels were similar ($95 \pm 8\%$) to those in N animals (100%), and both GLP-1 and Ex-4 treatments increased the value to 206 ± 30 and $239 \pm 30\%$ respectively ($P < 0.01$). OPG (both mRNA and protein) expression was lower in the HL rats than in the N group, and this downregulation was reversed by both GLP-1 and Ex-4 administration (Fig. 1). In contrast, RANKL (both gene and protein) expression in the HL rats was higher than that in the N group, and although GLP-1 failed to modify the RANKL mRNA value, Ex-4 treatment increased it even further; meanwhile, GLP-1, but not Ex-4, stimulated RANKL protein over that in the HL group. As a consequence, the OPG/RANKL ratio (both mRNA and protein) in the HL rats (Fig. 1) was lower than that in the N group, and it rose after GLP-1 and Ex-4 treatment, mainly accounted for by changes in OPG expression.

Bone mass analysis

Figure 2 shows the values of BMD and BMC in the femur and lumbar spine of HL rats, treated or untreated with GLP-1 or Ex-4, compared with those in the N group. At both the skeletal sites, BMD and BMC values in the HL group were significantly lower than those in the N rats, which were 168 ± 3 and 169 ± 7 mg/cm³ (femur) and 358 ± 16 and 546 ± 53 (vertebrae) respectively. Both GLP-1 and Ex-4 similarly reversed this decrease in BMD and BMC in the femur and vertebrae in these rats. At the latter site, the increase induced by both peptides tested on these bone mass parameters represented (overall mean) 21 ± 3 and $29 \pm 5\%$ HL value ($P < 0.001$) respectively.

Figure 2 Changes in BMD and BMC in the total femur and lumbar spine (L1–L4), of normal (N) and hyperlipidemic (HL) rats, untreated (grey) or treated with GLP-1 (black) or Ex-4 (white) as described in the text. Data (mean $\pm$ S.E.M.; $n = 8$ –12 rats) are expressed as percent increment of the value obtained in the N group. * $P < 0.05$ or lower versus N; # $P < 0.05$ or lower versus HL.

Table 2 Histomorphometric analysis of trabecular regions at the lumbar spine from normal (N) and hyperlipidemic (HL) rats, untreated or treated with GLP-1 or Ex-4. Data are mean $\pm$ s.e.m. ($n=5$ rats/group)

	N	HL	HL + GLP-1	HL + Ex-4
Static parameters				
BV/TV (%)	32.2 $\pm$ 4.6	19.2 $\pm$ 2.0*	31.8 $\pm$ 1.2 [†]	31.4 $\pm$ 2.1 [†]
Tb.Th (μ m)	527 $\pm$ 24	377 $\pm$ 22*	505 $\pm$ 18 [†]	737 $\pm$ 109 [†]
Tb.Sp (μ m)	1186 $\pm$ 202	1749 $\pm$ 94*	1092 $\pm$ 72 [†]	1422 $\pm$ 168
Tb.N (mm^{-1})	0.60 $\pm$ 0.06	0.47 $\pm$ 0.02	0.63 $\pm$ 0.04	0.49 $\pm$ 0.06
ES/BS (%)	13.8 $\pm$ 2.2	44.7 $\pm$ 5.5*	11.2 $\pm$ 0.5 [†]	13.3 $\pm$ 2.8 [†]
OcS/BS (%)	4.0 $\pm$ 1.2	20.7 $\pm$ 2.1*	2.2 $\pm$ 0.1 [†]	3.2 $\pm$ 0.9 [†]
n.Oc/TA (mm^2)	0.17 $\pm$ 0.03	0.48 $\pm$ 0.03*	0.11 $\pm$ 0.01 [†]	0.12 $\pm$ 0.03 [†]
MS/BS (%)	78.4 $\pm$ 4.7	78.6 $\pm$ 10.8	58.9 $\pm$ 2.5	71.2 $\pm$ 2.9
OV/TV (%)	18.9 $\pm$ 2.5	27.8 $\pm$ 5.3	5.4 $\pm$ 1.5 [†]	4.3 $\pm$ 0.4 [†]
OS/BS (%)	1.3 $\pm$ 0.1	1.8 $\pm$ 0.3	0.6 $\pm$ 0.2 [†]	1.0 $\pm$ 0.2
ObS/BS (%)	6.1 $\pm$ 1.1	11.0 $\pm$ 0.9*	4.8 $\pm$ 0.3 [†]	6.5 $\pm$ 0.9 [†]
Dynamic parameters				
MAR ($\mu\text{m}/\text{day}$)	0.39 $\pm$ 0.01	0.56 $\pm$ 0.07*	0.45 $\pm$ 0.02	0.36 $\pm$ 0.02 [†]
BFR/BS ($\mu\text{m}/\text{day}$)	0.34 $\pm$ 0.03	0.49 $\pm$ 0.06*	0.26 $\pm$ 0.02 [†]	0.27 $\pm$ 0.01 [†]

* $P < 0.05$ or lower versus N, [†] $P < 0.05$ or lower versus HL. BV/TV, bone volume per total tissue volume ratio; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; ES, erosive surface; BS, bone surface; OcS, osteoclast surface; n.Oc/TA, osteoclast number per total area ratio; MS, mineralized surface; OV, osteoid volume; OS, osteoid surface; ObS, osteoblast surface; MAR, mineral apposition rate; BFR, bone formation rate.

Bone histomorphometry

Alterations in the trabecular area of the lumbar spine were detected in the HL rats. These included a lower bone volume per total tissue volume ratio (BV/TV) and trabecular thickness (Tb.Th) values as well as a higher trabecular separation (Tb.Sp) (Table 2 and Fig. 3A), compared with those in the N rats. Furthermore, an increase in MAR and in BFR – together with an increased osteoblast surface (ObS)/BS – was observed in the lumbar vertebrae in these animals (Table 2). In fact, tetracycline double labels were more abundant than single labels in HL rats than in their controls

(Fig. 3B), which has probably resulted in a certain over-estimation of MAR in the former animals (Foldes *et al.* 1990). In addition, an increase in the number of erosive surfaces with abundant osteoclasts occurred at this skeletal site in this group of rats. Both GLP-1 and Ex-4 elicited a clear osteogenic action, as assessed by changes in BV/TV and several trabecular parameters at the lumbar spine of HL rats (Table 2 and Fig. 3A). This action is likely related to a decrease in bone remodeling; in fact, bone formation parameters normalized or even decreased below normal values in the lumbar vertebrae of HL rats after treatment with these peptides (Table 2 and Fig. 3B).

Figure 3 Light microscopy images showing the trabecular structure using von Kossa's staining (A), and single (arrowheads) and double (arrows) demeclocycline labels (B), in the lumbar vertebrae from representative normal (N) and hyperlipidemic (HL) rats, untreated or treated with GLP-1 or Ex-4. Original magnifications, $\times 400$.

Discussion

The old concept of bone and fat as inert metabolic tissues has been reconsidered in the light of recent findings showing that bone is able to modulate energy metabolism while adipokines exert an important influence on the mechanisms of bone mass maintenance (Lee *et al.* 2007). Moreover, the use of drugs for the treatment of metabolic disorders, such as type 2 diabetes and atherosclerosis, has shown to impact bone metabolism (Herrington & Potvin Klein 2001, Majima *et al.* 2007). In this regard, we recently reported that both GLP-1 and Ex-4 have osteogenic effects in two insulin resistance and type 2 diabetic rat models (Nuche-Berenguer *et al.* 2009, 2010a). In addition, the former incretin has been shown to directly interact with osteoblastic cells *in vitro* by interaction with a specific receptor different from that which is well characterized in the pancreas (Nuche-Berenguer *et al.* 2010b).

The HL model used in this study, showing the metabolic characteristics of obesity without changes in body weight, is of particular interest because it prevents the deleterious effects of hyperlipidemia from being overshadowed by a putative opposing action of body weight gain upon bone metabolism. Both femora and vertebrae displayed a diminished BMD and BMC and the OPG/RANKL ratio was reduced in the tibia in HL rats, suggesting a deleterious bone metabolism in this model. This was confirmed by performing bone histomorphometry in trabecular bone areas in the lumbar vertebrae of these rats, showing lower BV/TV and reduced Tb.Th, probably as a consequence of an increased bone resorption.

Recently, we showed that continuous infusion of GLP-1 and Ex-4 for 3 days into insulin-resistant or type 2 diabetic rats ameliorated the altered trabecular structure (evaluated by μ CT), related to an increase in OPG/RANKL gene ratio in the long bone (Nuche-Berenguer *et al.* 2009, 2010a). In this study, the same administration scheme of GLP-1 and Ex-4 into HL rats was shown to induce similar osteogenic effects as indicated by BMD and BMC increases in both femur and vertebrae, associated with changes in various trabecular structure parameters at the latter skeletal location. These changes were associated with the anti-resorptive features displayed by both peptides as depicted by bone histomorphometry and also by the observed changes in the OPG/RANKL ratio. Furthermore, as it was the case in the aforementioned T2D and IR models (Nuche-Berenguer *et al.* 2010a), Ex-4 was found to stimulate this ratio in HL rats even above than that in the normal group, also supporting the higher efficiency of Ex-4 with respect to GLP-1 in this model.

These aggregated findings, using different techniques to evaluate bone structure (μ CT and histomorphometry) and different rat models showing glucose and energy metabolism alterations, demonstrate that a short length of treatment with the tested peptides can exert positive effects on the trabecular bone at different skeletal sites. Our present data strongly suggest that the anti-resorptive properties of GLP-1,

consistent with those previously reported in a different experimental setting (Yamada *et al.* 2008), and that of its homologous peptide Ex-4 as shown here, are mainly responsible for their observed osteogenic action in HL rats.

It is currently intriguing whether these peptides might be able to directly target bone formation leading to anabolic effects in bone. Recent studies in diabetic rats (Nuche-Berenguer *et al.* 2009, 2010a) might support this notion, although an earlier report in knockout mice for the pancreatic GLP-1 receptor had suggested otherwise (Yamada *et al.* 2008). Also of interest in this regard, GLP-1, acting through the pancreatic GLP-1 receptor, has recently been shown to prevent the differentiation of human bone marrow stromal cells into adipocytes (Sanz *et al.* 2010); however, this study did not examine whether osteogenic differentiation might have been concomitantly induced by this peptide in these cells. Regarding the latter, we recently found that GLP-1 can directly upregulate OC gene expression in mouse osteoblastic MC3T3-E1 cells. It is presently unclear whether the effects of GLP-1 and Ex-4, as observed in this study and in our previous reports (Nuche-Berenguer *et al.* 2009, 2010a), are due to their interaction with different receptors and/or signaling in bone cells; in fact, a specific receptor for GLP-1, which is unrelated to the pancreatic GLP-1 receptor and does not bind Ex-4, has been described in osteoblastic cells (Nuche-Berenguer *et al.* 2010b).

In conclusion, the present findings indicate that this previously characterized HL model exhibits skeletal changes related to bone loss; thus, it can be a valuable model for studies to explore fat–bone relationships. Our findings also demonstrate that both GLP-1 and Ex-4 can reverse the bone alterations by inhibiting bone remodeling in HL rats, suggesting that these peptides could be envisioned as putative therapeutic agents to improve bone health in hyperlipidic states.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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