

The Murine Glucagon-Like Peptide-1 Receptor Is Essential for Control of Bone Resorption

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Gastrointestinal hormones including gastric inhibitory polypeptide (GIP), glucagon-like peptide (GLP)-1, and GLP-2 are secreted immediately after meal ingestion, and GIP and GLP-2 have been shown to regulate bone turnover. We hypothesize that endogenous GLP-1 may also be important for control of skeletal homeostasis. We investigated the role of GLP-1 in the regulation of bone metabolism using GLP-1 receptor knockout (Glp-1r^{-/-}) mice. A combination of bone density and histomorphometry, osteoclast activation studies, biochemical analysis of calcium and PTH, and RNA analysis was used to characterize bone and mineral homeostasis in Glp-1r^{-/-} and Glp-1r^{+/+} littermate controls. Glp-1r^{-/-} mice have cortical osteopenia and bone fragility by bone densitometry

as well as increased osteoclastic numbers and bone resorption activity by bone histomorphometry. Although GLP-1 had no direct effect on osteoclasts and osteoblasts, Glp-1r^{-/-} mice exhibited higher levels of urinary deoxyypyridinoline, a marker of bone resorption, and reduced levels of calcitonin mRNA transcripts in the thyroid. Moreover, calcitonin treatment effectively suppressed urinary levels of deoxyypyridinoline in Glp-1r^{-/-} mice and the GLP-1 receptor agonist exendin-4 increased calcitonin gene expression in the thyroid of wild-type mice. These findings establish an essential role for endogenous GLP-1 receptor signaling in the control of bone resorption, likely through a calcitonin-dependent pathway. (*Endocrinology* 149: 574–579, 2008)

BONE IS CONTINUOUSLY remodeled throughout life, and osteoblastic bone formation and osteoclastic bone resorption are closely coordinated by a variety of local and systemic factors to maintain constant bone mass. Bone resorption is known to be rapidly inhibited by acute nutrient ingestion, suggesting that it might be mediated by other physiological factors, the levels of which change in response to the nutritional state such as incretins. Gastrointestinal hormones including gastric inhibitory polypeptide (GIP), glucagon-like peptide (GLP)-1, and GLP-2 are secreted immediately upon meal ingestion, although the fasting level of these peptides is low. GIP and GLP-2 are known to be involved in the regulation of bone turnover (1, 2).

The effect of GIP on bone has been extensively investigated *in vitro* and *in vivo*. The GIP receptor is expressed in osteoblasts (3), and GIP increases collagen type 1 expression and alkaline phosphatase activity in osteoblast-like cells (3) and

protects osteoblasts from apoptosis (2), consistent with an anabolic effect. Recently, the presence of the GIP receptor in osteoclasts has been reported, and GIP has been shown to inhibit PTH-induced bone resorption, suggesting that a role of the postprandial rise in GIP is to stop active bone resorption such as occurs during fasting (4). The physiological importance of GIP receptor signaling on bone *in vivo* has been demonstrated using GIP receptor knockout (Gipr^{-/-}) mice, which exhibit a low bone mass phenotype due to both decreased bone formation and increased bone resorption (2, 5); and conversely, GIP-overexpressing transgenic mice exhibit increased bone mass (6).

GLP-2 is cosecreted with GLP-1 from L cells in the small and large intestine, and acts in the intestine to stimulate mucosal growth and nutrient absorption. Acute administration of GLP-2 decreases serum and urine markers of bone resorption in postmenopausal women (1, 7, 8), whereas bone formation appears to be unaffected by treatment with exogenous GLP-2. The effect of GLP-2 on bone has been investigated predominantly in humans, and the mechanism(s) underlying the GLP-2-mediated modulation of bone turnover remain unclear.

GLP-1 is well known as an incretin, and meal-stimulated plasma levels of GLP-1 are known to be diminished in patients with impaired glucose tolerance or type 2 diabetes (9). GLP-1 also has effects independent of insulin secretion such as inhibition of glucagon secretion and gastric emptying. In

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Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; BS, bone surface; BV, bone volume; CT, computed tomography; DPD, deoxyypyridinoline; ES, eroded surface; GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptide; N.Mu.Oc, number of multinuclear osteoclasts; N.Oc, number of osteoclasts; TV, tissue volume; WT, wild type.

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contrast to information derived from studies of GIP and GLP-2 on bone formation and resorption, the physiological role of GLP-1, if any, on bone is completely unknown. Because the GLP-1 receptor is expressed in thyroid C cells, and GLP-1 directly stimulates the secretion of calcitonin (10, 11), a potent inhibitor of osteoclastic bone resorption, GLP-1 may contribute to nutrient-mediated reduction of bone resorption.

In the present study, we have investigated the role of endogenous GLP-1 in the regulation of bone metabolism using GLP-1 receptor knockout (*Glp-1r*^{-/-}) mice. We performed morphological analyses of bones from *Glp-1r*^{-/-} mice and wild-type (WT) littermate controls, including densitometry and histomorphometry. We also evaluated the effects of exogenous GLP-1 on thyroid C cells, and we determined the effect of calcitonin treatment in *Glp-1r*^{-/-} mice. Taken together, our data illustrate an essential role for the GLP-1 receptor in the control of bone resorption.

Materials and Methods

Animals

Glp-1r^{-/-} mice and *Glp-1r*^{+/+} littermate WT controls were maintained on a C57BL/6 background as described previously (12). Mice were kept in cages with four to six animals per cage with free access to standard rodent diet and water. Male mice were used for all experiments. Crown to rump length was measured from tip of the nose to the end of the body. All procedures for animal care were approved by the Animal Care Committee of Kyoto University Graduate School of Medicine.

Bone densitometry and body composition analysis

For computed tomography (CT)-based analysis of bone mineral density (BMD), 10-wk-old WT and *Glp-1r*^{-/-} mice were anesthetized with ip injections of pentobarbital sodium (Nembutal; Dainippon Pharmaceutical, Osaka, Japan). Tibiae (between proximal and distal epiphysis) and lumbar spines (between L2 and L4) were scanned at 1-mm intervals using an experimental animal CT system (LaTheta LCT-100; Aloka, Tokyo, Japan). Bone mineral content (BMC) (milligrams), bone volume (cubic centimeters), and BMD (milligrams per cubic centimeter) were calculated using the LaTheta software (version 1.00). The minimum moment of inertia of cross-sectional areas (milligram-centimeters), which represents the flexural rigidity, and the polar moment of inertia of cross sectional areas (milligram-centimeters), which represents the torsional rigidity, were also calculated automatically by the LaTheta software (13). For body composition analysis, the whole bodies of 10-wk-old WT and *Glp-1r*^{-/-} mice were scanned using the LaTheta CT system.

Bone histomorphometry

Six-week-old male mice were used for studies of bone histomorphometry as described previously (2). Briefly, mice were double labeled with sc injections of 30 mg/kg tetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO) 4 d before being killed and 10 mg/kg calcein (Dojindo Co., Kumamoto, Japan) 2 d before being killed. Bones were stained with Villanueva bone stain for 7 d, dehydrated in graded concentrations of ethanol, and embedded in methyl-methacrylate (Wako Chemicals, Osaka, Japan) without decalcification. Bone histomorphometric measurements were made using a semiautomatic image analyzing system (System Supply, Ina, Japan) and a fluorescent microscope (Optiphot; Nikon, Tokyo, Japan) set at a magnification of $\times 400$. Standard bone histomorphometrical nomenclatures, symbols, and units were used as described in the report of the American Society of Bone and Mineral Research Histomorphometry Nomenclature Committee (14).

Osteoclast and osteoblast assays

For osteoclast differentiation assay, mouse primary osteoblasts and bone marrow cells were cocultured for 7 d in α -MEM (Sigma) containing

10% fetal bovine serum in the presence or absence of 10^{-8} M $1\alpha,25$ -dihydroxyvitamin D₃ with or without 10^{-5} M GLP-1 (Peptide Institute, Inc., Osaka, Japan). Cells positively stained for tartrate-resistant acid phosphatase containing more than three nuclei were counted as osteoclasts (15, 16). For pit formation assay of mature osteoclasts (16), aliquots of crude osteoclast preparations were plated on dentine slices and cultured with or without 10^{-4} M GLP-1 or 10^{-10} M calcitonin (Peptide Institute) for 48 h. The number of resorption pits was quantified under scanning electron microscopy. For osteoblast apoptosis assay, Saos-2 osteoblasts (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were pretreated for 1 h with or without 10^{-6} M GLP-1 and then incubated for an additional 6 h in the presence or absence of 50 μ M etoposide, as described previously (2).

Biochemical measurements

Total calcium concentration was measured using Spotchem SP-4420 (Arkray, Kyoto, Japan), and ionized calcium was measured using a blood gas analyzer (GEM premier 3000; Instrumentation Laboratory, Tokyo, Japan) after overnight fasting and 6 h after refeeding. Plasma insulin, leptin, and intact PTH levels were determined by ELISA kits for mouse insulin (Shibayagi, Gunma, Japan), mouse leptin (Morinaga, Yokohama, Japan) and mouse intact PTH (Immutopics Inc., San Clemente, CA). Urinary deoxyypyridinoline (DPD) concentrations were measured using an ELISA kit (Quidel, San Diego, CA) before and at 4 h after single administration of 10 IU/kg eel calcitonin (Elcitonin; Asahi Kasei Pharma, Tokyo, Japan).

RNA preparation and quantitative real-time PCR

For analysis of thyroid calcitonin gene expression, mice were injected ip with the GLP-1 receptor agonist exendin-4 (Sigma) at a dose of 24 nmol/kg or the same volume of PBS 6 h before RNA isolation. Total RNA was extracted from thyroid tissue using RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNAs were synthesized by SuperScript II Reverse Transcriptase system (Invitrogen, Carlsbad, CA) and subjected to quantitative real-time PCR using SYBR Green master kit and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers for the calcitonin gene were calcitonin forward 5'-CTCACCAG-GAAGGCATCAT-3' and calcitonin reverse 5'-CAGCAGGCGAATTCT-TCTT-3'. The relative amount of mRNA was calculated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as the invariant control: GAPDH forward 5'-TCGTTGATGGCAACAATCTC-3' and GAPDH reverse 5'-AAATGGTGAAGTCGGTGTG-3'.

Statistical analysis

Results are expressed as means \pm SE. Statistical significance was assessed by ANOVA and unpaired Student's *t* test, where appropriate. A *P* value of <0.05 was considered to be statistically significant.

Results

Baseline characteristics of WT and *Glp-1r*^{-/-} mice

Growth of *Glp-1r*^{-/-} mice was similar to that of WT mice in body weight during the 50-wk observation period (supplemental Fig. 1A, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Body length and length of tibia measured at 10 and 50 wk of age were also almost identical to each other (supplemental Fig. 1, B and C). No significant difference was observed in fat mass (supplemental Fig. 1D) and lean body mass (supplemental Fig. 1E) between 10-wk-old WT and *Glp-1r*^{-/-} mice determined by CT-based body composition analysis. Similarly, plasma leptin levels (supplemental Fig. 1F) were comparable in 10-wk-old WT and *Glp-1r*^{-/-} mice. These data indicate that there was no difference between WT and *Glp-1r*^{-/-} mice in body mass, body composition, or hormone levels that might affect bone mass.

Decreased cortical bone mass and diminished bone rigidity in the tibia of *Glp-1r^{-/-}* mice

To evaluate the impact of the lack of GLP-1 receptor signaling on bone mass, we performed CT-based bone densitometry in bones of differing cortical/cancellous bone ratio. Tibia and lumbar spine were used because the former has a higher cortical/cancellous bone ratio, whereas the latter has a lower cortical/cancellous bone ratio. The results are shown as total, cortical, cancellous, and trabecular bone mass in Fig. 1. There was no significant difference between WT and *Glp-1r^{-/-}* mice in BMC (milligrams) (Fig. 1, A–D) and bone volume (cubic centimeters) (Fig. 1, E–H). Total BMD of tibia was significantly lower in *Glp-1r^{-/-}* mice than in WT mice (WT mice, $612.97 \pm 4.03 \text{ mg/cm}^3$; *Glp-1r^{-/-}* mice, $570.07 \pm 4.22 \text{ mg/cm}^3$; $P = 0.0000036$), but no significant difference was observed in total BMD of spine (Fig. 2I). Cortical BMD also was significantly decreased in *Glp-1r^{-/-}* mice compared with WT mice in both tibia and spine (tibia: WT mice,

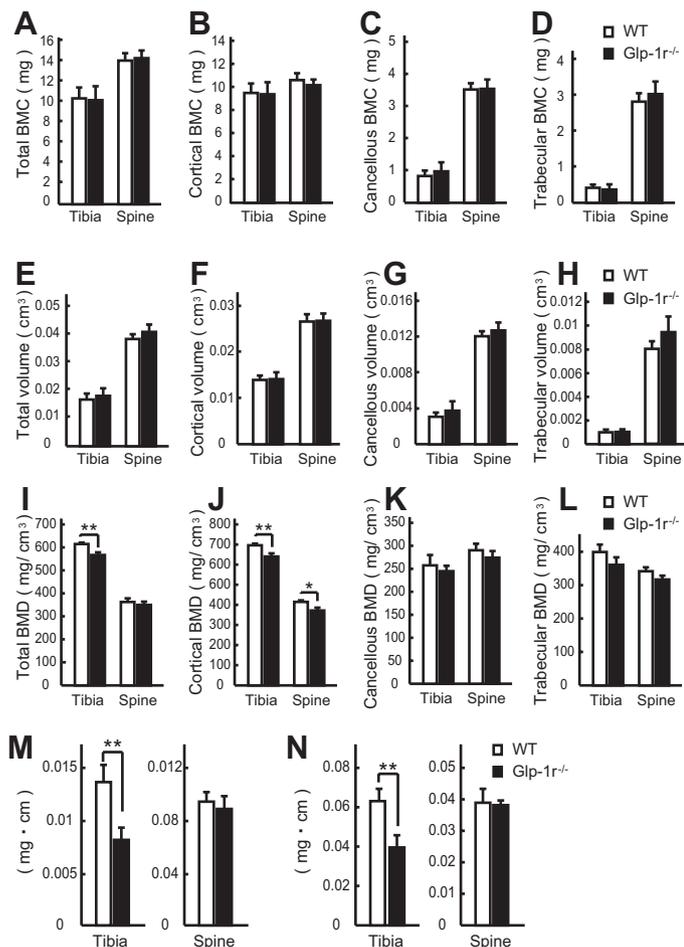


FIG. 1. CT-based bone densitometry of tibia and lumbar spine in 10-wk-old male WT (white bars) and *Glp-1r^{-/-}* (black bars) mice. A–D, Total (A), cortical (B), cancellous (C), and trabecular (D) BMC; E–H, total (E), cortical (F), cancellous (G), and trabecular (H) BV; I–L, total (I), cortical (J), cancellous (K), and trabecular (L) BMD; M, minimum moment of inertia of cross-sectional areas, representing the flexural rigidity; N, the polar moment of inertia of cross-sectional areas, representing the torsional rigidity, calculated by LaTheta software. Values are expressed as means \pm SE; $n = 6$ mice per group. *, $P < 0.05$; **, $P < 0.01$, WT vs. *Glp-1r^{-/-}* mice.

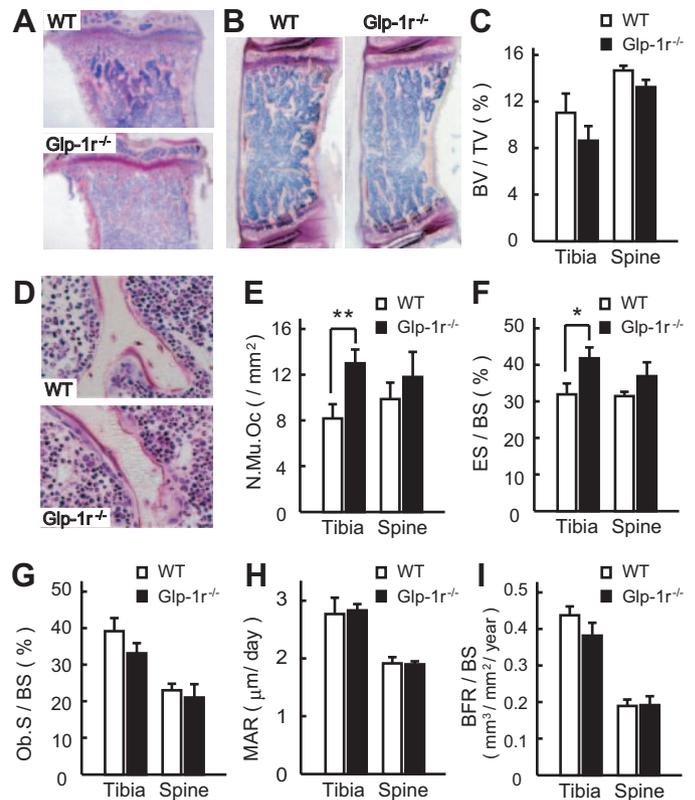


FIG. 2. Bone histomorphometry of tibia and lumbar spine in 6-wk-old male WT (white bars) and *Glp-1r^{-/-}* (black bars) mice. A, Representative pictures of proximal tibia. Original magnification, $\times 20$. B, Representative pictures of lumbar spine. Original magnification, $\times 40$. C, BV/TV of tibia and lumbar spine in WT and *Glp-1r^{-/-}* mice. D, Multinuclear osteoclasts in WT and *Glp-1r^{-/-}* mice. Original magnification, $\times 400$. E and F, N.Mu.Oc (E) and ES/BS (F) as cellular activity parameters regarding bone resorption. G–I, Osteoblast surface (Ob.S)/BS (G), mineral apposition rate (MAR) (H), and bone formation rate (BFR)/BS (I) as bone formation parameters. Values are expressed as means \pm SE; $n = 5$ –7 mice per group. *, $P < 0.05$; **, $P < 0.01$, WT vs. *Glp-1r^{-/-}* mice.

$687.34 \pm 3.57 \text{ mg/cm}^3$; *Glp-1r^{-/-}* mice, $650.06 \pm 10.59 \text{ mg/cm}^3$; $P = 0.0093$; spine: WT mice, $411.31 \pm 8.77 \text{ mg/cm}^3$; *Glp-1r^{-/-}* mice, $380.45 \pm 6.67 \text{ mg/cm}^3$; $P = 0.018$) (Fig. 1J). However, cancellous and trabecular BMD were not significantly different in WT and *Glp-1r^{-/-}* mice in both tibia and spine (Fig. 1, K and L). Reflecting the loss of cortical bone, *Glp-1r^{-/-}* mice showed skeletal fragility by diminished bone rigidity indexes. The minimum moment of inertia of cross-sectional areas, which represents flexural rigidity, was significantly reduced in *Glp-1r^{-/-}* mice (WT mice, $0.014 \pm 0.002 \text{ mg}\cdot\text{cm}$; *Glp-1r^{-/-}* mice, $0.008 \pm 0.001 \text{ mg}\cdot\text{cm}$; $P = 0.022$) (Fig. 1M). Moreover, torsional rigidity as indicated by the polar moment of inertia of cross-sectional areas also was significantly diminished in *Glp-1r^{-/-}* mice (WT mice, $0.064 \pm 0.006 \text{ mg}\cdot\text{cm}$; *Glp-1r^{-/-}* mice, $0.040 \pm 0.006 \text{ mg}\cdot\text{cm}$; $P = 0.020$) (Fig. 1N). These results indicate that *Glp-1r^{-/-}* mice have cortical osteopenia and bone fragility.

Glp-1r^{-/-} mice exhibit increased numbers of osteoclasts and bone resorption activity in the tibiae

We next performed histomorphometrical analyses of proximal tibiae (Fig. 2A) and lumbar spines (Fig. 2B) of 6-wk-old

male WT and *Glp-1r^{-/-}* mice. Although the bone volume (BV)/tissue volume (TV) ratio (Fig. 2C) was somewhat lower in *Glp-1r^{-/-}* mice in both tibia and spine, the difference was not statistically significant. The number of osteoclasts (N.Oc), especially multinuclear osteoclasts (N.Mu.Oc), the fully differentiated cells responsible for active bone resorption, was significantly increased in tibia of *Glp-1r^{-/-}* mice (Fig. 2, D and E), and all of the following parameters indicating osteoclastic number were also significantly higher in the tibia of *Glp-1r^{-/-}* mice: N.Mu.Oc per bone surface (BS) (2.06/mm² vs. 3.90/mm², $P = 0.022$), N.Mu.Oc per eroded surface (ES) (6.18/mm² vs. 9.32/mm², $P = 0.040$), N.Mu.Oc/TV (12.22/mm² vs. 20.26/mm², $P = 0.012$), N.Oc/BS (3.21/mm² vs. 5.98/mm², $P = 0.002$), and N.Oc/TV (19.28/mm² vs. 31.59/mm², $P = 0.009$), for WT vs. *Glp-1r^{-/-}* mice, respectively. Furthermore, eroded surface (ES/BS) was significantly increased in the tibiae of *Glp-1r^{-/-}* mice compared with WT mice (Fig. 2F). However, osteoclastic bone resorption activity was less apparent in spine of *Glp-1r^{-/-}* mice (Fig. 2, E and F). On the other hand, no significant difference was observed in bone formation parameters, including osteoblast surface per BS (Fig. 2G), mineral apposition rate (Fig. 2H), and bone formation rate (Fig. 2I) between WT and *Glp-1r^{-/-}* mice.

GLP-1 has no direct effect on osteoclasts and osteoblasts

Because osteoclastic number and bone resorptive activity were increased in *Glp-1r^{-/-}* mice, we investigated whether GLP-1 has a direct effect on osteoclasts and/or osteoblasts using cell culture models. We first evaluated the effect of GLP-1 on osteoclastic differentiation by culturing bone marrow cells together with osteoblasts, because osteoclasts are formed from the precursor cells in bone marrow by stimulation from osteoblasts. As a result, GLP-1 had no inhibitory effect on 1 α ,25-dihydroxyvitamin D₃-induced osteoclastic generation (Fig. 3A). Pit-forming assays showed that GLP-1 had no direct effect on pit-forming activity of mature oste-

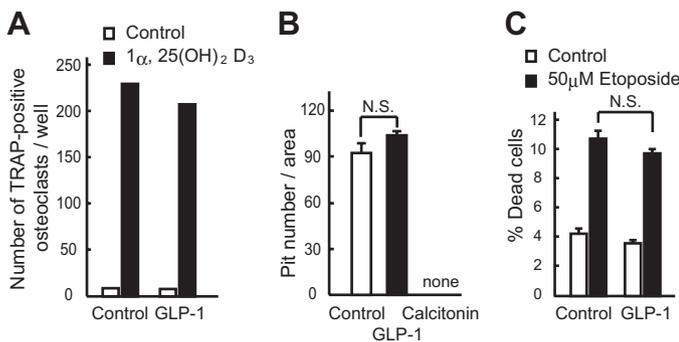


FIG. 3. Effects of GLP-1 on osteoclasts and osteoblasts *in vitro*. **A**, Effect of GLP-1 on osteoclastic differentiation. The numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts formed from coculture of osteoblasts and bone marrow cells in the presence or absence of 10⁻⁸ M 1 α ,25-dihydroxyvitamin D₃ [1 α , 25(OH)₂ D₃] (white bars) and/or 10⁻⁵ M GLP-1 (black bars) are shown. **B**, Effect of GLP-1 on the pit-forming activity of mature osteoclasts, using 10⁻¹⁰ M calcitonin as a positive control. **C**, Effect of GLP-1 on osteoblastic apoptosis. Saos-2 cells were pretreated with 10⁻⁴ M GLP-1 for 1 h and then incubated for an additional 6 h in the absence (white bars) or presence of 50 μ M etoposide (black bars). Values are expressed as means \pm SE.

oclasts placed on dentine slices, whereas calcitonin completely inhibited pit formation (Fig. 3B). Unlike the GIP receptor, the GLP-1 receptor was absent in osteoblasts, and GLP-1 failed to increase intracellular cAMP levels in Saos-2 cells (data not shown). Furthermore, GLP-1 had no protective effect on etoposide-induced osteoblastic apoptosis (Fig. 3C). These *in vitro* experiments demonstrate that GLP-1 has no direct effect on either osteoclasts or osteoblasts.

GLP-1 receptor signaling modulates calcitonin expression in mice

Because GLP-1 has no direct effect on bone cells, we investigated indirect pathways of GLP-1-mediated bone metabolism. Plasma levels of total calcium (data not shown) and ionized calcium (Fig. 4A) were unchanged in both fasting and fed conditions. Because hyperparathyroidism is a cause of cortical bone loss, plasma intact PTH levels were measured, but there was no difference in PTH levels between WT and *Glp-1r^{-/-}* mice (Fig. 4B). Because the GLP-1 receptor is expressed in thyroid C cells and GLP-1 stimulates calcitonin secretion *in vitro* via a cAMP-mediated mechanism (10, 11), calcitonin could be involved in the alteration of bone metabolism observed in *Glp-1r^{-/-}* mice. Quantitative real-time PCR analysis revealed that administration of the GLP-1 receptor agonist exendin-4 significantly increased thyroid calcitonin mRNA levels in WT mice (Fig. 4C). Conversely, the loss of GLP-1 receptor signaling in *Glp-1r^{-/-}* mice was as-

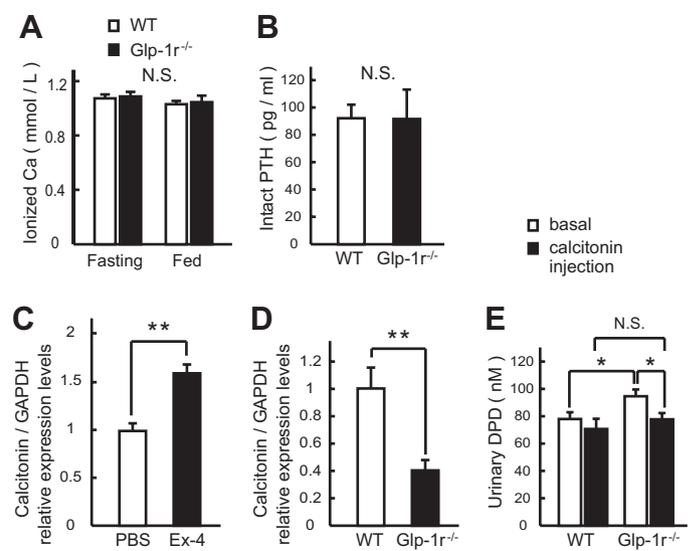


FIG. 4. Calcitonin deficiency resulted in increased bone resorption in *Glp-1r^{-/-}* mice. **A** and **B**, Plasma levels of ionized calcium (**A**) and intact PTH (**B**) in WT and *Glp-1r^{-/-}* mice. Values are expressed as means \pm SE; $n = 6$ –8 mice per group. **C**, Relative expression levels of calcitonin mRNA in thyroid from WT mice injected ip with PBS or 24 nmol/kg exendin-4 (Ex-4) 6 h before RNA isolation. Values are expressed as means \pm SE; $n = 5$ mice per group. *, $P < 0.01$, PBS vs. exendin-4 treatment. **D**, Relative expression levels of calcitonin mRNA in thyroid from WT and *Glp-1r^{-/-}* mice determined by quantitative real-time PCR. Values are expressed as means \pm SE; $n = 4$ mice per group. *, $P < 0.05$; **, $P < 0.01$, WT vs. *Glp-1r^{-/-}* mice. **E**, Urinary elimination of DPD from WT and *Glp-1r^{-/-}* mice before and at 4 h after single administration of 10 IU/kg calcitonin. Values are expressed as means \pm SE; $n = 6$ mice per group. *, $P < 0.05$, WT vs. *Glp-1r^{-/-}* mice.

sociated with a significant reduction in levels of calcitonin mRNA transcripts, 41% of levels in control WT thyroid glands (Fig. 4D). Consistent with results of bone histomorphometry showing increased osteoclastic bone resorption, *Glp-1r^{-/-}* mice showed significantly higher urinary DPD concentration (Fig. 4E). However, calcitonin treatment effectively decreased the urinary DPD concentration in *Glp-1r^{-/-}* mice (Fig. 4E), demonstrating that increased bone resorption in *Glp-1r^{-/-}* mice remains sensitive to the antiresorptive actions of calcitonin.

Discussion

Decreased BMD is a major determinant of fracture, but fracture risk in diabetic patients is often increased (17–19) and is not necessarily associated with decreased BMD. BMD in type 2 diabetes has been reported to be decreased, normal, or increased depending on various factors such as body weight or the site where BMD is measured. Body weight is one of the main determinants of BMD in both diabetic and nondiabetic subjects, suggesting that the increased BMD could be explained by the higher body weight. In the present study, there was no difference in several metabolic factors that often indirectly modulate BMD, including body weight, fat mass, or plasma levels of leptin, between WT and *Glp-1r^{-/-}* mice.

Quantitative CT was used in the present study for the measurement of BMD because of the merits of the method with regard to distinct assessment of cortical, cancellous, and trabecular bones and to providing indexes of bone strength in live animals (13, 20). We found that total BMD of tibia, which has a higher cortical/cancellous bone ratio, was significantly lower in *Glp-1r^{-/-}* mice and that cortical BMD at both tibia and lumbar spine was selectively reduced in *Glp-1r^{-/-}* mice compared with WT mice. Reflecting the cortical bone loss, *Glp-1r^{-/-}* mice showed skeletal fragility. In diabetic patients, BMD measured at sites with high cortical/cancellous bone ratio, such as distal radius or metacarpal bone, has been reported to be selectively decreased compared with sites high in cancellous bone such as lumbar spine or femoral neck (21–24). Reduced GLP-1 secretion is one of the features of type 2 diabetes (9), and it is of interest that cortical bone loss is observed in *Glp-1r^{-/-}* mice as well as in diabetic patients. Therefore, we suppose that modulation of GLP-1 receptor signaling may theoretically contribute to regulation of bone turnover in diabetic subjects, a hypothesis that requires further testing.

We found by bone histomorphometry that genetic loss of GLP-1 receptor signaling resulted in significantly increased osteoclastic bone resorption activity, whereas the effects on bone formation parameters were less marked, similar to the changes in bone turnover induced by gastrointestinal factors. However, unlike GIP, GLP-1 had no direct effects on osteoclasts and osteoblasts as shown by the *in vitro* experiments.

Calcitonin is a known inhibitor of bone resorption and has been reported to prevent or retard bone loss in animal models of excessive bone resorption (25–28). As to the effect of calcitonin on cortical bone, calcitonin treatment has been shown to increase lumbar vertebral cortical thickness (29) and femoral cortical areas (30) in ovariectomized rats. It has been

reported that the GLP-1 receptor is expressed in thyroid C cells and that GLP-1 stimulates calcitonin secretion via a cAMP-mediated mechanism in cultured C cells (10, 11); we also found that GLP-1 has a stimulatory effect on calcitonin gene expression in thyroid C cells *in vivo*, because attempts at measurement of plasma calcitonin were not successful due to sample volumes and assay sensitivity. Thus, increased osteoclastic bone resorption in *Glp-1r^{-/-}* mice might arise indirectly from loss of GLP-1 receptor signaling on C cells, leading to calcitonin deficiency. Consistent with this hypothesis, *Glp-1r^{-/-}* mice exhibit reduced levels of calcitonin mRNA transcripts in the thyroid. Furthermore, calcitonin treatment effectively suppressed the urinary DPD concentration in *Glp-1r^{-/-}* mice. Taken together, these findings are consistent with an essential role for calcitonin in the regulation of bone turnover (31) and raise the possibility that modulation of GLP-1 receptor signaling may regulate bone resorption indirectly through the thyroid C cell.

In summary, our present findings demonstrate that genetic disruption of GLP-1 receptor signaling results in cortical osteopenia and bone fragility due to increased bone resorption by osteoclasts, in association with reduced thyroid calcitonin expression. Moreover, exogenous GLP-1 administration increased calcitonin expression in the thyroid glands of normal WT mice. These findings raise the possibility that clinical modulation of GLP-1 receptor signaling in human subjects, either through administration of GLP-1 receptor agonists or dipeptidyl peptidase-4 inhibitors, may indirectly regulate bone turnover in diabetic subjects. Given the recent observations of reduced bone density and increased fracture rates in diabetic subjects treated with thiazolidinediones (32, 33), more studies directed at understanding the actions of therapies that activate GLP-1 receptor signaling seem warranted.

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