0013-7227/08/$15.00/0
Printed in U.S.A.

Endocrinology 149(2):574–579

doi: 10.1210/en.2007-1292

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

Chizumi Yamada, Yuichiro Yamada, Katsushi Tsukiyama, Kotaro Yamada, Nobuyuki Udagawa, Naoyuki Takahashi, Kiyoshi Tanaka, Daniel J. Drucker, Yutaka Seino, and Nobuya Inagaki

Department of Diabetes and Clinical Nutrition (C.Y., Y.Y., K.T., K.Y., Y.S., N.I.), Kyoto University Graduate School of Medicine, and Core Research for Evolutional Science and Technology of Japan Science and Technology Cooperation (N.I), Kyoto 606-8507, Japan; Department of Internal Medicine (Y.Y.), Division of Endocrinology, Diabetes and Geriatric Medicine, Akita University School of Medicine, Akita 010-8543, Japan; Department of Biochemistry (N.U.) and Institute for Oral Science (N.T.), Matsumoto Dental University, Nagano 399-0781, Japan; Department of Nutrition (K.T.), Kyoto Women's University, Kyoto 605-8501, Japan; The Samuel Lunenfeld Research Institute (D.J.D.), Department of Medicine, Mount Sinai Hospital and the Banting and Best Diabetes Center, University of Toronto, Toronto, Canada M5G 2C4; Kansai Electric Power Hospital (Y.S.), Osaka 553-0003, Japan

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 deoxypyridinoline, a marker of bone resorption, and reduced levels of calcitonin mRNA transcripts in the thyroid. Moreover, calcitonin treatment effectively suppressed urinary levels of deoxypyridinoline 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

First Published Online November 26, 2007

Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; BS, bone surface; BV, bone volume; CT, computed tomography; DPD, deoxypyridinoline; 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.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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 lumber 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 ×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 1α,25-dihydroxyvitamin D_3 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 μM 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 deoxypyridinoline (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′-CTCACCAGGAAGGCATCAT-3′ and calcitonin reverse 5′- CAGCAGGCGAACTTCTTCTT-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′-AAATGGTGAAGGTCGGTGTG-3′.

Statistical analysis

Results are expressed as means ± 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 lumber 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 ± 4.03 mg/cm^3; Glp-1r$^{-/-}$ mice, 570.07 ± 4.22 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, 687.34 ± 3.57 mg/cm^3; Glp-1r$^{-/-}$ mice, 650.06 ± 10.59 mg/cm^3; $P = 0.0093$; spine: WT mice, 411.31 ± 8.77 mg/cm^3; Glp-1r$^{-/-}$ mice, 380.45 ± 6.67 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 ± 0.002 mg·cm; Glp-1r$^{-/-}$ mice, 0.008 ± 0.001 mg·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 ± 0.006 mg·cm; Glp-1r$^{-/-}$ mice, 0.040 ± 0.006 mg·cm; $P = 0.020$) (Fig. 1N). These results indicate that Glp-1r$^{-/-}$ mice have cortical osteopenia and bone fragility.

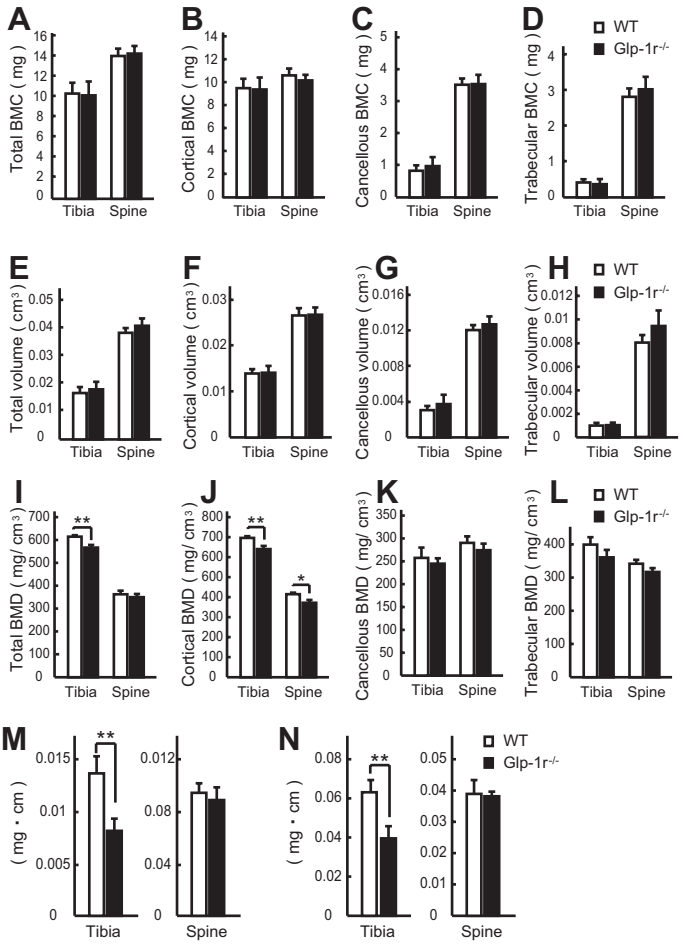

FIG. 1. CT-based bone densitometry of tibia and lumbar spine in 10-wk-old male WT (*white bars*) and Glp-1r$^{-/-}$ (*black bars*) mice. A–D, Total (A), cortical (B), cancellous (C), and trabecular (D) BMC; E–H, total (E), cortical (F), cancellous (G), and trabecular (H) BV; I–L, total (I), cortical (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 ± SE; n = 6 mice per group. *, $P < 0.05$; **, $P < 0.01$, WT *vs.* Glp-1r$^{-/-}$ mice.

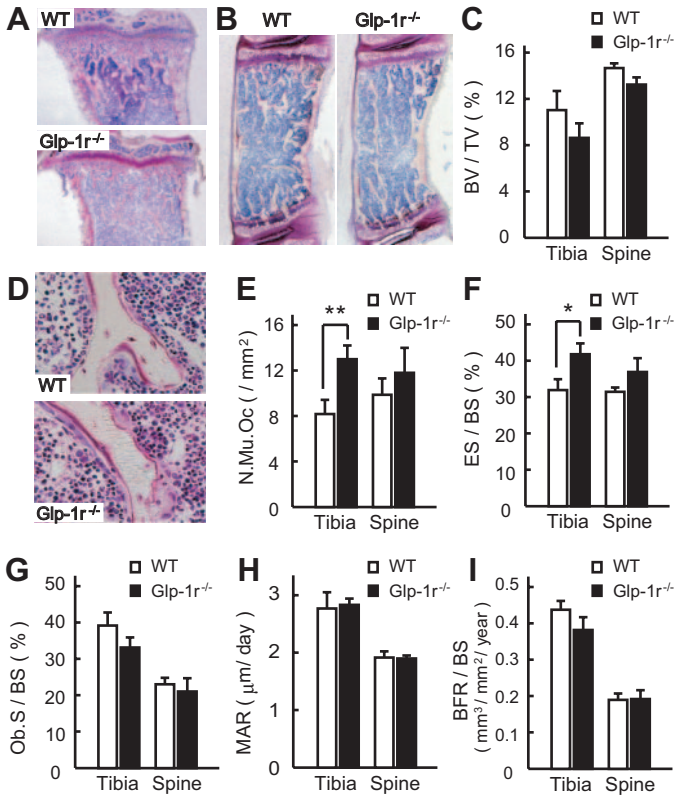

FIG. 2. Bone histomorphometry of tibia and lumbar spine in 6-wk-old male WT (*white bars*) and Glp-1r$^{-/-}$ (*black bars*) mice. A, Representative pictures of proximal tibia. Original magnification, ×20. B, Representative pictures of lumbar spine. Original magnification, ×40. C, BV/TV of tibia and lumbar spine in WT and Glp-1r$^{-/-}$ mice. D, Multinuclear osteoclasts in WT and Glp-1r$^{-/-}$ mice. Original magnification, ×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 ± SE; n = 5–7 mice per group. *, $P < 0.05$; **, $P < 0.01$, WT *vs.* Glp-1r$^{-/-}$ mice.

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/mm^2 *vs.* 3.90/mm^2, $P = 0.022$), N.Mu.Oc per eroded surface (ES) (6.18/mm^2 *vs.* 9.32/mm^2, $P = 0.040$), N.Mu.Oc/TV (12.22/mm^2 *vs.* 20.26/mm^2, $P = 0.012$), N.Oc/BS (3.21/mm^2 *vs.* 5.98/mm^2, $P = 0.002$), and N.Oc/TV (19.28/mm^2 *vs.* 31.59/mm^2, $P = 0.009$), 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 1α,25-dihydroxyvitamin D_3-induced osteoclastic generation (Fig. 3A). Pit-forming assays showed that GLP-1 had no direct effect on pit-forming activity of mature osteoclasts 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-1failed 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.

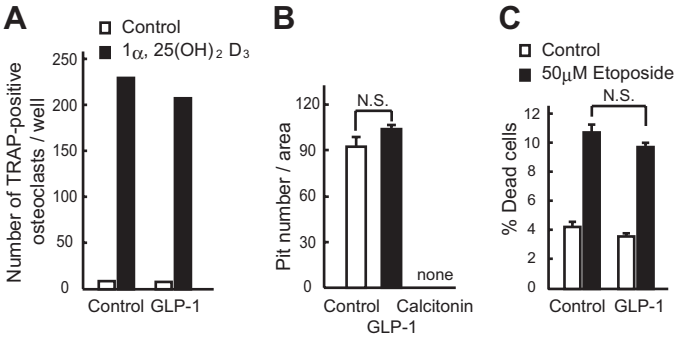

FIG. 3. Effects of GLP-1 on osteoclasts and osteoblasts *in vitro*. A, Effect of GLP-1 on osteoclastic differentiation. The numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts formed from coculture of osteoblasts and bone marrow cells in the presence or absence of 10^{-8} M 1α,25-dihydroxyvitamin D_3 [1α, 25$(OH)_2$ D_3] (*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 μM etoposide (*black bars*). Values are expressed as means ± SE.

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-

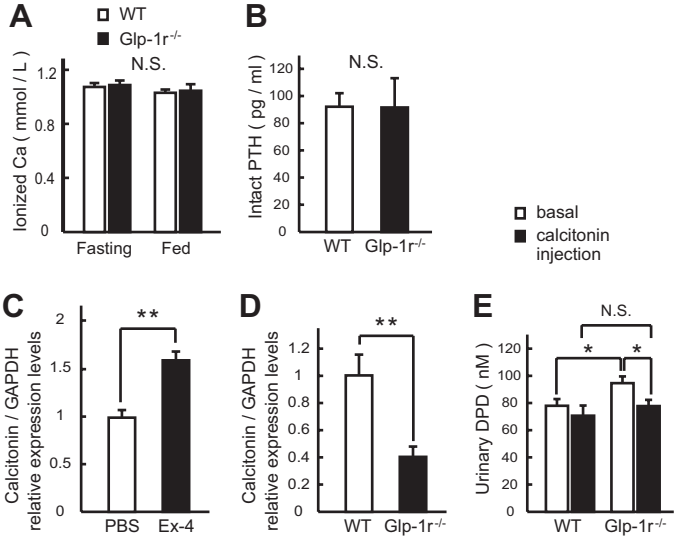

FIG. 4. Calcitonin deficiency resulted in increased bone resorption in Glp-1r$^{-/-}$ mice. A and B, Plasma levels of ionized calcium (A) and intact PTH (B) in WT and Glp-1r$^{-/-}$ mice. Values are expressed as means ± SE; n = 6–8 mice per group. C, Relative expression levels of calcitonin mRNA in thyroid 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 ± 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 ± 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 ± 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.

Acknowledgments

We gratefully acknowledge Ms. Akemi Ito, Niigata Bone Science Institute, for the measurement of bone histomorphometry, and also Dr. H. Yamauchi, Japan Osteoporosis Foundation, for technical advice on calcitonin experiments.

Received September 19, 2007. Accepted November 9, 2007.

Address all correspondence and requests for reprints to: Yuichiro Yamada, M.D., Ph.D., Department of Internal Medicine, Division of Endocrinology, Diabetes, and Geriatric Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita City, Akita 010-8543, Japan. E-mail: yamada@gipc.akita-u.ac.jp.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by Health Sciences Research Grants for Comprehensive Research on Aging and Health from the Ministry of Health, Labor, and Welfare, Japan; and by an operating grant from the Juvenile Diabetes Research Foundation, Canada, to D.J.D.

Present address for K.T.: Anjo Kosei Hospital, Japan.

Disclosure Statement: The authors have nothing to disclose.

References

1. **Henriksen DB, Alexandersen P, Bjarnason NH, Vilsboll T, Hartmann B, Henriksen EE, Byrjalsen I, Krarup T, Holst JJ, Christiansen C** 2003 Role of gastrointestinal hormones in postprandial reduction of bone resorption. J Bone Miner Res 18:2180–2189
2. **Tsukiyama K, Yamada Y, Yamada C, Harada N, Kawasaki Y, Ogura M, Bessho K, Li M, Amizuka N, Sato M, Udagawa N, Takahashi N, Tanaka K, Oiso Y, Seino Y** 2006 Gastric inhibitory polypeptide as an endogenous factor

promoting new bone formation after food ingestion. Mol Endocrinol 20:1644–1651
3. **Bollag RJ, Zhong Q, Phillips P, Min L, Zhong L, Cameron R, Mulloy AL, Rasmussen H, Qin F, Ding KH, Isales CM** 2000 Osteoblast-derived cells express functional glucose-dependent insulinotropic peptide receptors. Endocrinology 141:1228–1235
4. **Zhong Q, Itokawa T, Sridhar S, Ding KH, Xie D, Kang B, Bollag WB, Bollag RJ, Hamrick M, Insogna K, Isales CM** 2007 Effects of glucose-dependent insulinotropic peptide on osteoclast function. Am J Physiol Endocrinol Metab 292:E543–E548
5. **Xie D, Cheng H, Hamrick M, Zhong Q, Ding KH, Correa D, Williams S, Mulloy A, Bollag W, Bollag RJ, Runner RR, McPherson JC, Insogna K, Isales CM** 2005 Glucose-dependent insulinotropic polypeptide receptor knockout mice have altered bone turnover. Bone 37:759–769
6. **Xie D, Zhong Q, Ding KH, Cheng H, Williams S, Correa D, Bollag WB, Bollag RJ, Insogna K, Troiano N, Coady C, Hamrick M, Isales CM** 2007 Glucose-dependent insulinotropic peptide-overexpressing transgenic mice have increased bone mass. Bone 40:1352–1360
7. **Henriksen DB, Alexandersen P, Byrjalsen I, Hartmann B, Bone HG, Christiansen C, Holst JJ** 2004 Reduction of nocturnal rise in bone resorption by subcutaneous GLP-2. Bone 34:140–147
8. **Henriksen DB, Alexandersen P, Hartmann B, Adrian CL, Byrjalsen I, Bone HG, Holst JJ, Christiansen C** 2007 Disassociation of bone resorption and formation by GLP-2: a 14-day study in healthy postmenopausal women. Bone 40:723–729
9. **Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ** 2001 Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes 50:609–613
10. **Crespel A, De Boisvilliers F, Gros L, Kervran A** 1996 Effects of glucagon and glucagon-like peptide-1-(7–36) amide on C cells from rat thyroid and medullary thyroid carcinoma CA-77 cell line. Endocrinology 137:3674–3680
11. **Lamari Y, Boissard C, Moukhtar MS, Jullienne A, Rosselin G, Garel JM** 1996 Expression of glucagon-like peptide 1 receptor in a murine C cell line: regulation of calcitonin gene by glucagon-like peptide 1. FEBS Lett 393:248–252
12. **Hansotia T, Baggio LL, Delmeire D, Hinke SA, Yamada Y, Tsukiyama K, Seino Y, Holst JJ, Schuit F, Drucker DJ** 2004 Double incretin receptor knockout (DIRKO) mice reveal an essential role for the enteroinsular axis in transducing the glucoregulatory actions of DPP-IV inhibitors. Diabetes 53:1326–1335
13. **Yamanouchi K, Yada E, Hozumi H, Ueno C, Nishihara M** 2004 Analyses of hind leg skeletons in human growth hormone transgenic rats. Exp Gerontol 39:1179–1188
14. **Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR** 1987 Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2:595–610
15. **Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, Shinki T, Gillespie MT, Martin TJ, Higashio K, Suda T** 2000 Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 141:3478–3484
16. **Li X, Udagawa N, Itoh K, Suda K, Murase Y, Nishihara T, Suda T, Takahashi N** 2002 p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. Endocrinology 143:3105–3113
17. **Schwartz AV** 2003 Diabetes mellitus: does it affect bone? Calcif Tissue Int 73:515–519
18. **Carnevale V, Romagnoli E, D'Erasmo E** 2004 Skeletal involvement in patients with diabetes mellitus. Diabetes Metab Res Rev 20:196–204
19. **Rakic V, Davis WA, Chubb SA, Islam FM, Prince RL, Davis TM** 2006 Bone mineral density and its determinants in diabetes: the Fremantle Diabetes Study. Diabetologia 49:863–871
20. **Bagi CM, Hanson N, Andresen C, Pero R, Lariviere R, Turner CH, Laib A** 2006 The use of micro-CT to evaluate cortical bone geometry and strength in nude rats: correlation with mechanical testing, pQCT and DXA. Bone 38:136–144
21. **Christensen J O, Svendsen OL** 1999 Bone mineral in pre- and postmenopausal women with insulin-dependent and non-insulin-dependent diabetes mellitus. Osteoporos Int 10:307–311
22. **Suzuki K, Sugimoto C, Takizawa M, Ishizuka S, Kikuyama M, Togawa H, Taguchi Y, Nosaka K, Seino Y, Ishida H** 2000 Correlations between bone mineral density and circulating bone metabolic markers in diabetic patients. Diabetes Res Clin Pract 48:185–191
23. **Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, Hatanaka I, Nakao K** 2005 Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients. Osteoporos Int 16:907–913
24. **Suzuki K, Kurose T, Takizawa M, Maruyama M, Ushikawa K, Kikuyama M, Sugimoto C, Seino Y, Nagamatsu S, Ishida H** 2005 Osteoclastic function is accelerated in male patients with type 2 diabetes mellitus: the preventive role of osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG) on the decrease of bone mineral density. Diabetes Res Clin Pract 68:117–125
25. **Wronski TJ, Yen CF, Burton KW, Mehta RC, Newman PS, Soltis EE, DeLuca PP** 1991 Skeletal effects of calcitonin in ovariectomized rats. Endocrinology 129:2246–2250
26. **Li M, Shen Y, Burton KW, DeLuca PP, Mehta RC, Baumann BD, Wronski TJ** 1996 A comparison of the skeletal effects of intermittent and continuous administration of calcitonin in ovariectomized rats. Bone 18:375–380
27. **Wallach S, Rousseau G, Martin L, Azria M** 1999 Effects of calcitonin on animal and in vitro models of skeletal metabolism. Bone 25:509–516
28. **Mochizuki K, Inoue T** 2000 Effect of salmon calcitonin on experimental osteoporosis induced by ovariectomy and low-calcium diet in the rat. J Bone Miner Metab 18:194–207
29. **Mosekilde L, Danielsen CC, Gasser J** 1994 The effect on vertebral bone mass and strength of long term treatment with antiresorptive agents (estrogen and calcitonin), human parathyroid hormone-(1–38), and combination therapy, assessed in aged ovariectomized rats. Endocrinology 134:2126–2134
30. **Giardino R, Fini M, Aldini NN, Gnudi S, Biagini G, Gandolfi MG, Mongiorgi R** 1996 Calcitonin and alendronate effects on bone quality in osteoporotic rats. J Bone Miner Res 21:S335 (Abstract)
31. **Huebner AK, Schinke T, Priemel M, Schilling S, Schilling AF, Emeson RB, Rueger JM, Amling M** 2006 Calcitonin deficiency in mice progressively results in high bone turnover. J Bone Miner Res 21:1924–1934
32. **Schwartz AV, Sellmeyer DE, Vittinghoff E, Palermo L, Lecka-Czernik B, Feingold KR, Strotmeyer ES, Resnick HE, Carbone L, Beamer BA, Park SW, Lane NE, Harris TB, Cummings SR** 2006 Thiazolidinedione use and bone loss in older diabetic adults. J Clin Endocrinol Metab 91:3349–3354
33. **Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G** 2006 Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N Engl J Med 355:2427–2443

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**