Chronic Reduction of GIP Secretion Alleviates Obesity and Insulin Resistance Under High-Fat Diet Conditions.

Nasteska, Daniela; Harada, Norio; Suzuki, Kazuyo; Yamane, Shunsuke; Hamasaki, Akihiro; Joo, Erina; Iwasaki, Kanako; Shibue, Kimitaka; Harada, Takanari; Inagaki, Nobuya

Nasteska, Daniela ...[et al]. Chronic Reduction of GIP Secretion Alleviates Obesity and Insulin Resistance Under High-Fat Diet Conditions.. Diabetes 2014, 63(7): 2332-2343

2014-07

http://hdl.handle.net/2433/189090

© 2014 by the American Diabetes Association.; Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.
Daniela Nasteska, Norio Harada, Kazuyo Suzuki, Shunsuke Yamane, Akihiro Hamasaki, Erina Joo, Kanako Iwasaki, Kimitaka Shibue, Takanari Harada, and Nobuya Inagaki

Chronic Reduction of GIP Secretion Alleviates Obesity and Insulin Resistance Under High-Fat Diet Conditions

Diabetes 2014;63:2332–2343 | DOI: 10.2337/db13-1563

Gastric inhibitory polypeptide (GIP) exhibits potent insulino tropic effects on β-cells and anabolic effects on bone formation and fat accumulation. We explored the impact of reduced GIP levels in vivo on glucose homeostasis, bone formation, and fat accumulation in a novel GIP-GFP knock-in (KI) mouse. We generated GIP-GFP KI mice with a truncated prepro-GIP gene. The phenotype was assessed in heterozygous and homozygous states in mice on a control fat diet and a high-fat diet (HFD) in vivo and in vitro. Heterozygous GIP-GFP KI mice (GIP-reduced mice [GIPgfp/+]) exhibited reduced GIP secretion; in the homozygous state (GIP-lacking mice [GIPgfp/gfp]), GIP secretion was undetectable. When fed standard chow, GIPgfp/+ and GIPgfp/gfp mice showed mild glucose intolerance with decreased insulin levels; bone volume was decreased in GIPgfp/gfp mice and preserved in GIPgfp/+ mice. Under an HFD, glucose levels during an oral glucose tolerance test were similar in wild-type, GIPgfp/+ and GIPgfp/gfp mice, while insulin secretion remained lower. GIPgfp/+ and GIPgfp/gfp mice showed reduced obesity and reduced insulin resistance, accompanied by higher fat oxidation and energy expenditure. GIP-reduced mice demonstrate that partial reduction of GIP does not extensively alter glucose tolerance, but alleviates obesity and lessens the degree of insulin resistance under HFD conditions, suggesting a potential therapeutic value.

Gastric inhibitory polypeptide (GIP) is a 42–amino acid polypeptide produced by enteroendocrine K cells, which are located mainly in the upper parts of the small intestine. Its main secretagogues are glucose and, even more intensely, fats that reach the intestinal lumen soon after food intake (1). Following secretion, the hormone exerts its effects through specific, G-protein-coupled receptors located mainly in the stomach, pancreas, central nervous system, bone, and adipose tissue (2,3). Apart from its role in the inhibition of gastric acid secretion (4), GIP exhibits potent glucose-dependent insulino tropic action (5,6), and, therefore, it is classified as an incretin (3). In addition to its insulino tropic effect, in the absence of which glucose intolerance develops (7), GIP stimulates islet growth (8) and proliferation of β-cells (9), and reduces β-cell apoptosis (10,11). Studies of GIP receptor (GIPR) knock-out (GIPRKO) mice (7) describe GIP as an obesity-promoting factor in high-fat diet (HFD) conditions, and show that deletion of GIPR signaling causes resistance to obesity (12) but leads to osteoporosis (13), revealing an important role of GIP in bone metabolism. However, in these studies, as well as in a model of GIPR antagonism (14), the reported changes were focused on disrupted or blocked GIPR signaling. The condition of reduced GIP secretion and how it affects the pancreatic and extrapancreatic effects of GIP remain unclear.

The aim of the current study is to explore the potential of reduced GIP levels in vivo, and to define the impact on glucose homeostasis, bone formation, and fat accumulation in a novel GIP–green fluorescent protein (GFP) knock-in (KI) mouse model characterized by truncation of the prepro-GIP gene and insertion of a GFP sequence (15). The model was developed for the purpose of visualization and identification of K cells and exhibits reduced...
or absent GIP secretion in heterozygous GIP-reduced mice and homozygous or GIP-lacking mice, respectively. Establishing the phenotype of the heterozygous GIP-reduced mouse is important to understand the possible benefits of a limited reduction of GIP secretion.

**RESEARCH DESIGN AND METHODS**

**Animals**

Male GIP-GFP KI mice and wild-type (WT) littermates were used in all experiments. GIP-GFP KI mice were generated as described previously (15). The animals were maintained under conditions of a 12 h light/dark cycle, with free access to water and food, unless indicated otherwise. Starting from 7 weeks of age, the mice were divided into the following two groups: the control fat diet (CFD) group, receiving food with 10% of fat and energy density of 3.8 kcal/g (catalog no. D12450B; Research Diets Inc., New Brunswick, NJ); and the HFD group, receiving food with 60% of fat and energy density of 5.2 kcal/g (catalog no. D12492; Research Diets Inc.). In total, six groups of mice (five to six mice per group) were used throughout the study: WT mice on CFD, heterozygous GIP-GFP KI mice (GIP<sup>gfp/gfp</sup>) on CFD, homozgyous GIP-GFP KI mice (GIP<sup>gfp/gfp</sup>) on CFD, WT on HFD, GIP<sup>gfp/+</sup> mice on HFD, and GIP<sup>gfp/gfp</sup> on HFD. After 8 weeks of CFD or HFD feeding, the animals were used in the experiments listed below. Maintenance of the mice and all experimental procedures were approved by Kyoto University Animal Care Committee.

**Expression Levels of GIPR mRNA**

After standard chow feeding or at least 8 weeks of CFD and HFD feeding, mice were killed by cervical dislocation, and the pancreas and white (visceral) adipose tissue were harvested. The white adipose tissue was frozen immediately in liquid nitrogen and stored at −80°C until further use; the pancreas was digested using the collagenase method, and islets were obtained. Islet mRNA (RNasy Lipid Tissue Mini Kit; Qiagen, Hilden, Germany) and adipose tissue mRNA (RNasy Lipid Tissue Mini Kit; Qiagen) were extracted and cDNA was synthesized by reverse transcription (SuperScript II; Invitrogen, Carlsbad, CA). GIPR mRNA expression levels were quantified by semiquantitative real-time PCR (AB StepOne Plus Real Time PCR; Applied Biosystems, Foster City, CA) using GIPR forward and reverse primers with the following sequence: 5′-CCTCCACTGGTCCCTACAC-3′ (forward primer) and 5′-GATAAAACCTCCACCAGTAG-3′ (reverse primer). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The sequences of GAPDH forward and reverse primers are as follows: 5′-AAATGGTGAGGTGTTGTTGTG-3′ for the forward primer, and 5′-TGGTTGGATGCGAAATCTCTC-3′ for the reverse primer.

**Measurement of GIP Content and Protein Content**

Mice were killed at 6 weeks of age by cervical dislocation, intestine samples were taken and washed in PBS, weighed, and, after overnight extraction with 5 mL/g acid ethanol (at 4°C), GIP content was measured by ELISA (Millipore Corp., Billerica, MA). Protein content was measured using Bradford Protein Assay (Bio-Rad, Hercules, CA). In brief, dye reagent was diluted, and protein (albumin) standards were made in duplicate. Standards and intestine samples were loaded on a microtiter plate, incubated at room temperature for 5 min, and absorbance was measured at 595 nm. GIP content was expressed as GIP content per protein content.

**Bone Histomorphometry**

Six-week-old mice that had been fed standard chow were prepared for bone histomorphometry measurement by subcutaneous injection of 25 mg/kg tetracycline hydrochloride (Sigma-Aldrich, St. Louis, MO) 4 days before they were killed and 10 mg/kg calcine (Dojindo, Kumamoto, Japan) 2 days before they were killed. Animals were killed by cervical dislocation, and tibiae were removed and fixed with 70% ethanol. Further processing of tibia samples (muscle removing, dehydration in graded concentration of ethanol, Villanueva bone staining, and embedding in methyl methacrylate), preparation of frontal plane sections of tibiae, and bone histomorphometry measurement using a semiautomatic image-analyzing system (System Supply, Nagano, Japan) were performed by Niigata Bone Science Institute, Niigata, Japan.

**Oral Glucose Tolerance Test and Measurement of Hormones**

Following 8 weeks of CFD and HFD, the mice underwent an oral glucose tolerance test (OGTT). The fasting period (overnight fasting) was begun 19 h prior to the experiment. During the test, blood samples were obtained by heparinized microcapillary tubes from the orbital sinus of the mice at the following time intervals: 0 min (fasting levels), and 15, 30, 60, and 120 min after glucose administration. Glucose (2 g/kg in mice on standard chow and 1 g/kg in mice on HFD) was given orally, using a gavage tube. Blood glucose levels were measured by the glucose oxidase method (Sanwa Kagaku Kenkyusho, Nagoya, Japan). After collection, blood samples were kept on ice and then centrifuged (3,000 rotations per minute for 10 min at 4°C), and serum was separated. The serum samples were used fresh or kept at −80°C until further processing. Insulin, total GIP, and total glucagon-like peptide 1 (GLP-1) levels were measured by ELISA as follows: insulin kit (Shibayagi, Shibukawa, Japan), total GIP kit (Millipore, Billerica, MA), and total GLP-1 kit (Meso Scale Discovery, Rockville, MD).

**Insulin Tolerance Test**

The mice were fasted 4–6 h before the start of the experiment. Blood samples were drawn from the orbital sinus using heparinized microcapillary tubes at the following time intervals: 0 min (fasting levels), and 15, 30, 60, and 120 min after insulin administration. Human insulin (100 units/mL; Eli Lilly and Co., Indianapolis, IN) was...
administered intraperitoneally in a dose of 0.5 units/kg. Blood glucose levels were measured by the glucose oxidase method (Sanwa Kagaku Kenkyusho).

**Measurement of Body Fat Composition (Measurement of Subcutaneous and Visceral Fat)**
In young mice at the age of 7 weeks, or after 8 weeks of feeding with a CFD or HFD, body fat was measured by a computed tomography (CT) scan (A La Theta LCT-100; Hitachi Aloka, Tokyo, Japan). The mice were anesthetized with intraperitoneal injection of sodium pentobarbital and placed in a measurement chamber of the CT scanner in the supine position. The scanned area of the body was flanked by the xiphisternum and sacrum; the width of scanned slices was 2 mm. The images obtained were analyzed using A La Theta software, version 1.00, and values for body fat, both subcutaneous and visceral, were quantified in grams.

**Indirect Calorimetry and Mice Activity**
Mice were kept 6 – 7 weeks on CFD or HFD, and afterward indirect calorimetry was performed and the activity of the mice was measured (ARCO 2000 mass spectrometer; Arco System, Chiba, Japan). Each mouse was placed in an individual chamber with free access to water and CFD or HFD. Respiratory quotient, energy expenditure (in calories per minute per kilogram), fat oxidation (in milligrams per minute per kilogram), and mice activity (in counts per minute) were measured every 5 min over 48 h.

**In Vitro Insulin Secretion**
For the measurement of glucose-stimulated insulin secretion in vitro, islets from mice on CFD and HFD were isolated using collagenase digestion method. In brief, mice were killed by cervical dislocation; 0.5 mg/mL collagenase dissolved in Hanks’ balanced salt solution was injected through the bile duct into the pancreas, and, after its expansion, it was manually isolated and incubated in Krebs-Ringer bicarbonate buffer (KRBB; 120 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.2 mmol/L KH2PO4, 2.4 mmol/L CaCl2, and 20 mmol/L NaHCO3) at 37°C over 21 min. After homogenizing the pancreas with KRBB, the islets were separated by centrifugation in Ficoll gradient. Separated islets were resuspended in KRBB on a dish and handpicked under a light microscope. For glucose-stimulated insulin secretion assessment, three batches with different glucose concentrations were prepared, as follows: 5.5 mmol/L glucose, 11.1 mmol/L glucose, and 11.1 mmol/L glucose plus 100 nmol/L GIP-human (Peptide Institute, Osaka, Japan). For each sample containing 500 μL incubation medium (KRBB; 2 mol/L HEPES, pH 7.4; 0.2% BSA), 10 islets were handpicked in a volume of 200 μL KRBB and incubated at 37°C during 30 min (following preincubation in the same conditions). For the measurement of insulin content in islets of HFD-fed mice, samples were incubated overnight with 5 mL/g acid ethanol (at 4°C). Insulin concentration and insulin content were measured using radioimmunoassay (Aloka Accuflex γ 7000; Hitachi, Tokyo, Japan).

**Measurement of β-Cell Area**
Whole pancreas was isolated manually from mice kept on CFD and HFD for 8 weeks. All isolated organs were fixed in Bouin’s solution, then were washed with 50% ethanol once per day over 1 week, and, finally, embedded in paraffin. Every fifth section of the pancreas was used for analysis. In total, three sections (slides) per pancreas (per mouse) were analyzed. The paraffin slides were deparaffinized with lemosol, rehydrated with 100% and 70% ethanol, blocked by 3% peroxidase, incubated overnight (at 4°C) in a humidified chamber with polyclonal rabbit anti-insulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and conjugated with fluorescent secondary antibody the next day. After immunostaining, all slides were analyzed by immunofluorescent microscope (Keyence Corp., Osaka, Japan) using BZ Analyzer software. The area of the whole pancreas and the area of insulin-immunopositive cells were measured at the same time. The β-cell area was expressed as β-cell area/total pancreas area in all analyzed slides.

**Statistics**
All results are expressed as the mean ± SE. Statistical analyses were performed using ANOVA with Tukey test, and P values <0.05 were considered statistically significant.

**RESULTS**

**GIP Reduction in GIP-GFP KI Mice**
The main genetic trait of GIP-GFP KI mice is alteration (truncation) of the prepro-GIP gene coupled with insertion of the GFP coding sequence (15). In mice kept on standard chow, the assessment of GIP mRNA levels in the small intestine showed reduced levels in GIP<sup>gfp/+</sup> mice (P < 0.05), while in GIP<sup>gfp/gfp</sup> mice, GIP mRNA could not be detected (P < 0.05, P < 0.01) (Fig. 1A). Small intestine GIP contents were reduced in GIP<sup>gfp/+</sup> mice (P < 0.05) and were undetectable in GIP<sup>gfp/gfp</sup> mice (P < 0.01) when compared with WT mice. Total GIP levels during OGTT (Fig. 1B) as well as GIP secretion (as shown by the area under the curve of GIP) (Fig. 1C) were reduced by ~50% in GIP<sup>gfp/+</sup> mice (P < 0.01) and were below the lower limit of detection in GIP<sup>gfp/gfp</sup> mice (P < 0.001) compared with control WT mice (the lower detection limit of ELISA total GIP levels kit was 8.2 pg/mL).

**Body Weight Progression, Glucose Tolerance, and β-Cell Profile Following GIP Reduction in Standard Chow-Fed Mice**
Starting from 4 weeks of age, the body weight of weaning mice fed standard chow (containing 10% fat) was recorded, and no changes were seen among WT, GIP<sup>gfp/+</sup>, and GIP<sup>gfp/gfp</sup> mice (Fig. 2A). The measurement of body fat composition (body fat) in the 7th week of age (just before placing the mice on an HFD) revealed similar amounts of body fat in all mice (Fig. 2B). During an OGTT, blood glucose levels were higher in GIP<sup>gfp/+</sup> mice than those in WT mice at 30 min (P < 0.05), whereas in GIP<sup>gfp/gfp</sup>
mice glucose elevation persisted at 30 and 60 min (P < 0.05) (Fig. 2C). Meanwhile, insulin levels in both GIP<sup>+/+</sup> and GIP<sup>−/−</sup> mice remained lower in comparison with WT, especially at 15 and 30 min after glucose load (P < 0.05) (Fig. 2D). In vitro measurement of insulin secretion (Fig. 2E) showed a similar pattern of secretion in all types of mice at 5.5 mmol/L glucose, as well as at 11.1 mmol/L glucose. When challenged with 100 nmol/L human GIP peptide together with 11.1 mmol/L glucose, the islets of GIP-GFP KI mice, both GIP<sup>+/+</sup> and GIP<sup>−/−</sup>, exhibited a similar insulin response to that of WT mice. GIPR mRNA levels in β-cells remained unchanged in GIP-GFP KI mice when compared with controls (Fig. 2F). mRNA expression of preproglucagon, peptide YY, cholecystokinin, somatostatin, and secretin in the small intestine showed no differences in GIP-GFP KI mice compared with WT mice (data not shown). Plasma GLP-1 levels during the OGTT did not differ among the three types of mice (WT mice 15.54 ± 6.8 pg/mL, GIP<sup>+/+</sup> mice 11.83 ± 4.97 pg/mL, GIP<sup>−/−</sup> mice 18.54 ± 3.96 pg/mL at 15 min after OGTT).

Body weight follow-up of mice on CFD in a period of 8 weeks (starting at the age of 7 weeks) showed that the body weight progression did not differ among all three groups of mice (Supplementary Fig. 1A). The overall glucose response to 0.5 units/kg human insulin (insulin tolerance test [ITT] data) was similar in WT, GIP<sup>+/+</sup>, and GIP<sup>−/−</sup> mice at all time points of the experiment (at 60 min of ITT, GIP<sup>+/+</sup> mice had lower glucose levels when compared with WT mice (Supplementary Fig. 1B).

**Bone Formation in Conditions of Standard Chow Feeding**
Following GIP reduction, the bone volume in GIP<sup>+/+</sup> mice was similar to that in WT mice, whereas GIP<sup>−/−</sup> mice had reduced bone volume (P < 0.05) (Fig. 3A). Furthermore, the number of trabeculae in GIP<sup>+/+</sup> mice showed no changes when compared with WT mice, while GIP<sup>−/−</sup> mice exhibited a decrease (P < 0.05) (Fig. 3C), as demonstrated by the images of proximal tibial sections (Fig. 3B). Although osteoblast surface was decreased in GIP<sup>+/+</sup> mice compared with WT mice (P < 0.05) (Fig. 3D), the bone formation rate (Fig. 3F) remained unchanged in these mice. The osteoclast surface was increased in GIP<sup>−/−</sup> mice when compared with WT mice, while in GIP<sup>+/+</sup> mice it remained similar to WT mice (Fig. 3E).

**Induction of Metabolic Stress by HFD**
Figures 4 and 5 describe the phenotype changes induced by HFD feeding for 8 weeks (56 days). Starting from the second week of HFD feeding, WT mice steadily increased their body weight (P < 0.001) compared with the lean control (Fig. 4A), while within the HFD group, GIP<sup>+/+</sup> mice showed less body weight gain (P < 0.01 at 2nd week; P < 0.001 at 8th week) than WT mice; GIP<sup>−/−</sup> mice exhibited the lowest body weight gain (P < 0.001 at 2nd week; P < 0.001 at 8th week vs. WT HFD). Ad libitum glucose levels were measured at the same time, once per week, and the overall glucose levels in all mice remained similar (Fig. 4B). Food and water intake were similar in all groups of mice (data not shown).

During OGTT, total GIP levels and GIP secretion were increased twofold in WT mice on HFD (P < 0.001) compared with the lean control; in HFD-fed mice, GIP<sup>+/+</sup> mice exhibited decreased levels (P < 0.05), while GIP<sup>−/−</sup> mice showed an absence of GIP (P < 0.001) (Fig. 4C and D). Fasting glucose levels in WT mice on HFD were higher (P < 0.01) when compared with their lean littermates; on HFD background, glucose levels remained similar in all mice (Fig. 4E). The overall insulin response (Fig. 4F) in WT mice on HFD was more intense than that of the control mice, and, within the HFD group, the insulin levels of GIP<sup>+/+</sup> mice remained lower in comparison with those of WT mice (P < 0.05), while GIP<sup>−/−</sup> mice showed the lowest insulin levels (P < 0.01, P < 0.001). Insulin secretion in vitro (Fig. 4G) was similar among all mice on CFD and HFD in the presence of 5.5 mmol/L glucose. In response to 11.1 mmol/L glucose, WT mice on HFD had higher insulin secretion compared with WT mice on CFD, whereas in the HFD group similar levels were found in WT and GIP<sup>+/+</sup> mice, coupled with lower
insulin levels in GIPf/gfp mice (P < 0.05 vs. WT HFD, P < 0.01 vs. GIPf/+). When 100 nmol/L human GIP peptide was added to 11.1 mmol/L glucose, insulin secretion remained lower in GIPf/gfp mice on HFD (P < 0.01). Measurement of the β-cell area, as expressed by the ratio of β-cell area to total pancreas area, showed a tendency toward an increase observed in HFD-fed mice, relative to the lean mice, although the difference was not statistically significant. However, within the HFD group, β-cell area remained similar in WT and GIPf/+ mice, while GIPf/gfp mice exhibited decreased β-cell area (P < 0.05) (Fig. 4H). The expression levels of GIPR mRNA in the islets were similar in WT mice on CFD and HFD, as well as in GIPf/+ mice on HFD, whereas in GIPf/gfp mice levels were reduced (P < 0.01 vs. GIPf/+)(Fig. 4I).

Adipose Tissue Response to HFD Feeding and Consequential Energy Expenditure Changes

CT scan measurement (Fig. 5A) of visceral, subcutaneous, and total body fat demonstrated a large increase in fat accumulation in WT mice on HFD (P < 0.01) when compared with the lean mice. On an HFD background, WT
mice accumulated more body fat than their GIP<sup>+/+</sup> littermates (<i>P</i> < 0.05), while the fat depots in GIP<sup>+/−</sup>/GIP<sup>−/−</sup> were greatly reduced (<i>P</i> < 0.01), showing levels similar to the lean control. The CT scan images of abdominal sections of mice on CFD and HFD visualize the difference in fat accumulation among all groups. Assessment of insulin resistance by ITT (Fig. 5B) showed a rise in glucose levels in HFD-fed WT mice compared with lean mice, while within the HFD group a better response to insulin was observed in both GIP<sup>+/−</sup> and GIP<sup>+/−/−</sup> mice, with glucose levels remaining lower (<i>P</i> < 0.05; <i>P</i> < 0.01) compared with WT mice. In relation to these data, a tendency toward increased fat oxidation (Fig. 5C) in all mice on HFD was observed (<i>P</i> < 0.05), with a larger increase in GIP<sup>+/−</sup> mice (<i>P</i> < 0.05), especially in the dark phase, and even higher in GIP<sup>/+−/−</sup> mice (<i>P</i> < 0.05). In addition, the energy expenditure measurement on HFD background (Fig. 5D) showed an increase in GIP<sup>+/−</sup> mice (<i>P</i> < 0.05) (again, more prominent in the dark phase) and in GIP<sup>/+−/−</sup> mice (<i>P</i> < 0.05) when compared with WT mice. Concomitantly, mice activity was measured (Fig. 5E), and no statistically significant changes were found in the HFD group. Expression levels of GIPR mRNA in white (visceral) adipose tissue (Fig. 5F) remained unchanged in all animals on CFD and HFD, except for GIP<sup>/+−/−</sup> mice, in which the levels were elevated (<i>P</i> < 0.05).

**DISCUSSION**

Studies in single and double incretin receptor knock-out mice (16) have shown that, although secretion of GIP and GLP-1 is triggered by different factors, they have an additive stimulating effect on β-cells with regard to insulin secretion, with GIP accounting for the larger portion of the total incretin effect in male mice. Furthermore, human data demonstrated that after an oral glucose load of 75 g and a mixed meal load (17,18), secretion of GIP is more pronounced than GLP-1 secretion, suggesting that GIP may play a more potent role in the regulation of postprandial insulin secretion in nondiabetic conditions. We have generated GIP-GFP KI mice characterized by truncation of the prepro-GIP gene and insertion of the GFP coding sequence that leads to reduced GIP production in heterozygous state and the absence of GIP production in the homozygous state. GIP<sup>+/−</sup> mice exhibit a phenotype similar to that of GIPRKO mice regarding glucose tolerance, bone formation, and adipose tissue expansion (Table 1). However, GIP<sup>/+−/−</sup> mice represent a novel mouse model in which GIP, despite its secretion being reduced by half, maintains glucose levels similar to those of controls (Fig. 4B and E) and lessens insulin resistance in mice with HFD-induced obesity (Figs. 4F and 5B).

When fed standard chow, GIP<sup>/+−/−</sup> mice, in a manner similar to GIPRKO mice, had higher glucose excursions accompanied with insufficient production of insulin during OGTT (Table 1). Despite having reduced, but still present, GIP secretion, GIP<sup>/+−/−</sup> mice also showed mild glucose intolerance and lower insulin secretion, confirming the potent insulinotrophic effect of GIP (Fig. 2C and D, and Table 1). Furthermore, insulin secretion tests in vitro
Figure 4—Induction of metabolic stress by HFD. Body weight (A) and ad libitum glucose levels (B) in WT CFD, WT HFD, GIPgfp/+ HFD, and GIPgfp/gfp HFD mice were measured once per week during 8 weeks (56 days) of feeding with CFD (10% of fat) or HFD (60% of fat). Total GIP levels (C), GIP secretion (GIP area under the curve) (D), glucose levels (E), and insulin levels (F) were measured during OGTT (glucose load of 1 g/kg body weight) conducted after 8 weeks (56 days) of CFD or HFD feeding. G: In vitro insulin secretion from isolated islets was measured in conditions of 5.5 mmol/L glucose, 11.1 mmol/L glucose, and 11.1 mmol/L glucose plus 100 nmol/L human GIP. Results were expressed as insulin secretion (% insulin content). H: β-Cell area was measured by immunohistochemistry of pancreas sections and subsequent analysis using BZ Analyzer software. Results are expressed as β-cell area/total pancreas area. I: GIPR mRNA levels in islets were expressed as GIPR mRNA/GAPDH mRNA. *n = 5–6 mice or samples per group; 10 islets per sample. WT CFD mice are represented by white circles with square dot dash and white bars with square dot border, WT HFD mice are represented by white circles with solid dash and white bars with solid border, GIPgfp/+ mice are represented by black squares and black bars, and GIPgfp/gfp mice are represented by gray triangles and gray bars. P values are expressed as follows: A, C–F: *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT HFD; B: *P < 0.05 WT CFD vs. WT HFD; and #P < 0.05 GIPgfp/+ HFD vs. WT HFD. G–I: *P < 0.05, **P < 0.01, ***P < 0.001. The absence of an asterisk above the horizontal brackets in G–I indicates no statistical significance.
demonstrated a similar pattern of secretion in all groups of mice. The measurement of mRNA expression levels of GIPR in the islets showed no changes among all groups of mice, indicating the presence of functional GIPRs.

Similar to GIP-lacking and GIP-reduced mice, rat GIP promoter-diphtheria toxin A chain transgenic mice exhibit glucose intolerance, in their case very profound, with complete abolition of the incretin effect, and show similarities in phenotype under HFD conditions (19). In this mouse model (GIP promoter-diphtheria toxin A chain), forced expression of attenuated diphtheria toxin was established under the rat GIP promoter, leading to isolated
Table 1—Phenotype comparison of GIP-GFP KI mice and GIPRKO mice

<table>
<thead>
<tr>
<th>Genotype/phenotype</th>
<th>GIP&lt;sup&gt;gfp/+&lt;/sup&gt;</th>
<th>GIP&lt;sup&gt;gfp/+&lt;/sup&gt; HFD</th>
<th>GIP&lt;sup&gt;gfp/+&lt;/sup&gt;</th>
<th>GIP&lt;sup&gt;gfp/+&lt;/sup&gt; HFD</th>
<th>GIPRKO</th>
<th>GIPRKO HFD (6,12,29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard chow feeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIP secretion</td>
<td>↓ (−50%)</td>
<td>Absent</td>
<td>Absent</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose tolerance</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Impaired</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone volume</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFD feeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose tolerance</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>Body weight</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>Fat mass</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

Standard chow feeding data are relative to WT; HFD feeding data are relative to WT HFD. ↔, no changes; ↓, decreased; ↓↓, highly decreased; ↑, increased; ↑↑, highly increased.

In addition to its insulinoergic role, GIP is involved in modulation of bone formation. There are GIP-specific receptors located on osteoblasts (25) and osteoclasts (26). GIP operates as an anabolic hormone in the bone, where it stimulates incorporation of meal-derived Ca<sup>2+</sup> into bone and bone building (13), and reduces bone absorption by inhibiting osteoclastic activity. Studies in GIPRKO mice have shown that the absence of GIPR signaling leads to significant osteoporosis due to lower osteoblast and higher osteoclast action (13). Similar to GIPRKO mice, GIP-lacking mice also showed signs of osteoporosis, manifested by reduced bone volume, reduced number of trabeculae, and increased osteoclast surface. On the other hand, GIP-reduced mice maintained normal bone volume and bone trabeculae, and, despite the exhibited reduction of osteoblast surface, no increased osteoclast activity was observed. More importantly, the bone formation rate remained normal, indicating that reduction of GIP by ~50% does not significantly impair the beneficial role of GIP in bone formation. Considering the glucose intolerance of these mice, it appears that reduction of GIP secretion more profoundly affects the insulin-potentiating role of GIP, indicating differing regulatory mechanisms of GIP action in β-cells and in bone (Table 1).

To better understand the extent of the phenotypic consequences following GIP reduction, we induced chronic metabolic stress by feeding the mice with HFD. Previous reports indicate a strong connection between GIP secretion and obesity in HFD-feeding conditions (27). High caloric intake causes hypersecretion of GIP (12,28,29) due to hyperexpression of the GIP gene (15) and a subsequent rise in insulin secretion (30), leading to increased fat deposition in the adipose tissue and expansion of fat depots (31,32). GIP increases the adipose tissue volume directly (33,34) by binding to its receptors located on the adipocytes and indirectly by potentiating β-cell secretion of insulin, which is known to be involved in adipocyte fat deposition (35). In our study, HFD feeding for at least 8 weeks resulted in the absence of circulating GIP levels in GIP-lacking mice (consistent with data from standard chow-fed mice), whereas in GIP-reduced
mice, the reduction of GIP was similar to the levels observed in lean WT mice and was lower when compared with WT mice on HFD. The most obvious consequence of HFD was a change in body weight and fat mass in WT and GIP-GFP KI mice (Figs. 4A and 5A, and Table 1). Although WT mice showed overt obesity, GIP-lacking mice retained their body weight and fat mass at levels similar to those of their lean littermates, as previously reported in GIPRKO mice, in mice with K-cell ablation (19) and in mice with chemical inhibition of GIPR signaling (14). GIP-reduced mice also maintained lower body weight throughout the experiment period of 56 days, indicating that while the lack of body weight gain and fat mass was not as evident as it was in GIP-lacking mice, the reduced obesity was nevertheless important. Moreover, the glucose response to insulin during ITT showed lower levels in both GIP-reduced and GIP-lacking mice fed HFD, indicating that not only complete but also partial reduction of GIP alleviates insulin resistance while reducing obesity. Thus, a reduction of GIP secretion as shown in GIP-reduced mice mitigates both direct and indirect actions of GIP on adipose tissue and leads to reduced diet-induced obesity.

Glucose levels measured ad libitum throughout most of the experiment period were similar in WT mice and GIP-GFP KI mice, and glucose excursions during OGTT remained similar as well in all mice under HFD conditions. However, all HFD-fed mice had higher OGTT glucose levels when compared with the lean controls, suggesting that when metabolic stress was introduced, glucoregulation was similarly achieved in conditions of normal and reduced GIP production. At the same time, the circulating insulin levels were lower in GIP-lacking and GIP-reduced mice than those of WT mice on HFD, accompanied by decreased insulin resistance (Fig. 5B and Table 1). These data are in line with those of a previous study (36) examining the role of insulin in obesity and type 2 diabetes. These findings are potentially promising for a new therapeutic approach to obesity and type 2 diabetes.

In conclusion, our data suggest that the reduction of GIP secretion in vivo confirms the potent role of GIP in insulin secretion and leads to reduced obesity and reduced insulin resistance in HFD conditions without severely impairing glucose homeostasis and without disrupting the role of GIP in bone formation. These findings are especially in the dark phase, although without a statistically significant difference.

In conclusion, our data suggest that the reduction of GIP secretion in vivo confirms the potent role of GIP in insulin secretion and leads to reduced obesity and reduced insulin resistance in HFD conditions without severely impairing glucose homeostasis and without disrupting the role of GIP in bone formation. These findings are especially promising for a new therapeutic approach to obesity and type 2 diabetes.

Acknowledgments. The authors thank Shoichi Asano and Dr. Xibao Liu from the Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, for their technical support regarding the study. The authors thank Shoichi Asano and Dr. Xibao Liu from the Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, for their technical support regarding the study. Funding. This study was supported by Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and from the Ministry of Health, Labor, and Welfare, Japan. Duality of Interest. N.I. served as a medical advisor for Takeda, Taisho Pharmaceutical, GlaxoSmithKline, Mitsubishi Tanabe Pharma; lectured for Meso Scale Discovery, Sanofi, Novartis Pharma, Dainippon Sumitomo Pharma, Kyowa Kirin, and Mitsubishi Tanabe Pharma; and received payment for his services. N.I
received a clinical commission/joint research grant from Mesa Scale Discovery, Eli Lilly Japan, Shiratori Pharmaceutical, Roche Diagnostics, and the Japan Diabetes Foundation; and also received a scholarship grant from Mesa Scale Discovery, Japan Tobacco Inc., Nippon Boehringer Ingelheim, Takeda, Danippon Sumitomo Pharma, Astellas Pharma, Daichii-Sankyo, and Mitsubishi Tanabe Pharma. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. D.N. researched the data; contributed to the discussion; and wrote, reviewed, and edited the manuscript. N.H. and N.I. contributed to the discussion, and reviewed and edited the manuscript. K.Su., S.Y., E.J., K.I., K.Sh., and T.H. contributed to the discussion. N.I. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of the study were presented in abstract form at the 47th Annual Meeting of the European Association for the Study of Diabetes, Lisbon, Portugal, 12–16 September 2011; at the 48th Annual Meeting of the European Association for the Study of Diabetes, Berlin, Germany, 1–5 October 2012; and at the 73rd Scientific Sessions of the American Diabetes Association, Chicago, IL, 21–25 June 2013.

References
25. Pacheco-Pantoja EL, Ranganath LR, Gallager JA, Wilson PJM, Fraser WD. Receptors and effects of gut hormones in three osteoblastic cell lines. BMC Physiol 2011;11:12


