これまで集積した糖尿病家族歴濃厚大家系2家系、累計25名に関して、ゲノムDNA抽出用採血後も継続的に臨床データのフォローアップを継続している。本施設で見いだした糖尿病家系が京都大学で解析され、糖尿病関連候補遺伝子を絞り込む基盤となった (Mol. Genet. Metab.109(1):112-117, 2013)。当院では、継続的に当該患者親族の他の構成メンバーの本研究参加に関するリクルートを進めている。

D．考察

糖尿病病態や発症関連遺伝子に関する人種差の報告から、日本人検体を用いた本研究は有意義なものと考えられる。上記、糖尿病発症原因候補遺伝子絞り込みまで解析し得た家系も大家系であり、本研究戦略上、糖尿病発症大家系の集積が特に重要であると思われる。患者および親族フォローアップは長期間に及ぶことになるため十分なインフォームドコンセントと互いの信頼関係構築が重要であると思われた。今後も家系探索を継続予定である。

E．結論

継続的に日本人糖尿病発症原因遺伝子同定のため、家族歴濃厚家系の収集を行い、3世代にわたり複数の糖尿病患者を含む糖尿病家族歴濃厚家系を3家系見いだし、臨床データを集積している。当院で発見し研究参加承諾された糖尿病大家系検体を用いて京都大学でゲノム解析し、糖尿病関連候補遺伝子が絞り込めた(Mol. Genet. Metab.109(1):112 -117, 2013)。親族の追加研究参加を募るとともに新規適合家系の検索も進めていく予定である。

F．健康危険情報

特になし

G．研究発表

1. 論文発表

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2. 学会発表

なし

H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3.その他
なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Kondo Y, Toyoda K, Santo T, Fujii J, Fukushima M, Inagaki N, Yasuda K.	A patient who developed symptomatic reactive hypoglycemia 14 years after total gastrectomy and was successfully treated with miglitol.	***Diabetol Int.***	4	66-70	2013

Chronic reduction of GIP secretion alleviates obesity and insulin resistance under high fat diet condition

Running title: GIP reduction alleviates obesity

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Abstract:

Gastric inhibitory polypeptide (GIP) exhibits potent insulinotropic 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 state in mice on control fat diet (CFD) and high fat diet (HFD) *in vivo* and *in vitro*. Heterozygous GIP-GFP KI mice (GIP-reduced mice: $GIP^{gfp/+}$) exhibited reduced GIP secretion; in homozygous state (GIP-lacking mice: $GIP^{gfp/gfp}$), GIP secretion was undetectable. When fed standard chow, $GIP^{gfp/+}$ and $GIP^{gfp/gfp}$ showed mild glucose intolerance with decreased insulin levels; bone volume was decreased in $GIP^{gfp/gfp}$ and preserved in $GIP^{gfp/+}$. Under HFD, glucose levels during OGTT were similar in WT, $GIP^{gfp/+}$, and $GIP^{gfp/gfp}$, while insulin secretion remained lower. $GIP^{gfp/+}$ and $GIP^{gfp/gfp}$ 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 alter extensively glucose tolerance, but it alleviates obesity and lessens the degree of insulin resistance under HFD condition, suggesting a potential therapeutic value.

Keywords: GIP, incretin, bone volume, obesity, insulin resistance

Abbreviations:

GIP gastric inhibitory polypeptide

GLP-1 glucagon-like polypeptide-1

GIPR GIP receptor

GIPRKO GIP receptor knockout

GIP-GFP KI gastric inhibitory polypeptide-green fluorescent protein knock-in

CFD control fat diet

HFD high fat diet

$GIP^{gfp/+}$ heterozygous GIP-GFP KI

$GIP^{gfp/gfp}$ homozygous GIP-GFP KI

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 insulinotropic action (5, 6), and, therefore, it is classified as an incretin (3). In addition to its insulinotropic 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 knockout (GIPRKO) mice (7) describe GIP as an obesity-promoting factor in high fat diet (HFD) conditions, and show that deletion of GIP receptor (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 in a model of GIP receptor 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 present 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-GFP knock-in (KI) mouse model characterized by truncation of the prepro-GIP gene and insertion of a Green Fluorescent Protein (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 limited reduction of GIP secretion.

Research design and methods

Animals. Male GIP-GFP KI mice and wild-type littermates (WT) were used in all experiments. GIP-GFP KI mice were generated as described previously (15). The animals were maintained in conditions of 12 h of light cycle and 12 h of dark cycle, with free access to water and food, unless indicated otherwise. Starting from 7 weeks of age, the mice were divided into two groups: control fat diet (CFD) group, receiving food with 10% of fat and energy density of 3.8 kcal/g (Research Diets Inc. New Brunswick, NJ, USA; cat.no.D12450B), and high fat diet (HFD) group, receiving food with 60% of fat and energy density of 5.2 kcal/g (Research Diets Inc. New Brunswick, NJ, USA; cat.no.D12492). In total, 6 groups of mice (5-6 mice per group) were used throughout the study: WT on CFD, heterozygous GIP-GFP KI mice ($GIP^{gfp/+}$) on CFD, homozygous GIP-GFP KI mice ($GIP^{gfp/gfp}$) on CFD, WT on HFD, heterozygous GIP-GFP KI mice ($GIP^{gfp/+}$) on HFD and homozygous GIP-GFP KI mice ($GIP^{gfp/gfp}$) on HFD. After 8 weeks of control fat or high fat 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 GIP receptor (GIPR) mRNA. After standard chow feeding or at least 8 weeks of CFD and HFD, mice were sacrificed by cervical dislocation, and 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; pancreas was digested using collagenase method and islets were obtained. Islet mRNA (RNeasy Mini Kit, Qiagen, Hilden, Germany) and adipose tissue mRNA (RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany) were extracted

and cDNA (complementary DNA) was synthesized by reverse transcription (SuperScript II, Invitrogen, NY, USA). GIPR mRNA expression levels were quantified by semi-quantitative real-time polymerase chain reaction (RT-PCR) (Applied Biosystems, AB StepOne Plus Real Time PCR, Foster City, CA, USA) using GIPR forward and reverse primer with the following sequence: 5'-CCTCCACTGGGTCCCTACAC-3'(forward primer) and 5'-GATAAACACCCTCCACCAGTAG-3' (reverse primer). GAPDH mRNA was used as an internal control. The sequences of GAPDH forward and reverse primer are as follows: 5'-AAATGGTGAAGGTCGGTGTG-3' for the forward primer and 5'-TCGTTGATGGCAACAATCTC-3' for the reverse primer.

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

Bone histomorphometry. Mice at the age of 6 weeks kept on standard chow were prepared for bone histomorphometry measurement by injecting subcutaneously 25 mg/kg of tetracycline hydrochloride (Sigma-Aldrich, St.Louis, MO, USA) 4 days before sacrifice and 10 mg/kg of calcein (Dojindo, Kumamamoto, Japan) 2 days before sacrifice. Animals were sacrificed by cervical dislocation and tibiae were removed and fixed with 70% ethanol. Further processing of tibiae samples (muscle removing, dehydration in graded concentration of ethanol, Villanueva

bone staining and embedding in methyl-metacrylate), preparation of frontal plane sections of tibiae and bone histomorphometry measurement using semiautomatic image analyzing system (System Supply, Nagano, Japan) were done by Niigata Bone Science Institute, Niigata, Japan.

Oral glucose tolerance test (OGTT) and measurement of hormones. Following 8 weeks of CFD and HFD, the mice underwent oral glucose tolerance test (OGTT). The fasting period (overnight fasting) was begun 19 h prior to the experiment. During the test, blood samples were taken by heparinized micro-capillary tubes from the orbital sinus of the mice at the following time intervals: 0 min (fasting levels), 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 gavage tube. Blood glucose levels were measured by glucose oxidase method (Sanwa Kagaku Kenkyusho CO. LTD., Nagoya, Japan). After collecting the blood samples, they were kept on ice, and then centrifuged (3000 rpm/10 min/4^{o}C) and serum was separated. The serum samples were used fresh or kept at -80^{o}C until further processing. Insulin, total GIP, and total GLP-1 levels were measured by ELISA as follows: insulin kit (Shibayagi, Gumma, Japan), total GIP kit (Millipore Corp, Bilerica, MA, USA), and total GLP-1 kit (MSD, Meso Scale Discovery, Rockville, MD, USA).

Insulin tolerance test (ITT). The mice were fasted 4-6 h before the start of the experiment. Blood samples were drawn from the orbital sinus using heparinized micro-capillary tubes at the following time intervals: 0 min (fasting levels), 15, 30, 60, and 120 min after insulin administration. Human insulin (100 U/ml, Eli Lilly and Co, Indianapolis, IN, USA) was administered intraperitoneally in a dose of 0.5 U/kg. Blood glucose levels were measured by glucose oxidase method (Sanwa Kagaku Kenkyusho CO. LTD., Nagoya, Japan).

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 CFD or HFD, body fat was measured by computerized tomography (CT) scan (A La Theta LCT-100, Aloka, Tokyo, Japan). The mice were anesthetized with intraperitoneal injection of sodium pentobarbital and placed in a measurement chamber of the CT scanner in supine position. The scanned area of the body was flanked by xiphisternum and sacrum; the width of scanned slices was 2 mm. Obtained images were analyzed using A La Theta software, version 1.00 and values of body fat, both subcutaneous and visceral, were quantified in grams (g).

Indirect calorimetry and mice activity. Mice were kept 6-7 weeks on CFD or HFD and, afterwards, indirect calorimetry was performed and activity of the mice was measured (ARCO 2000 Mass spectrometer-ARCOSYSTEM Inc, Chiba, Japan). Each mouse was placed in an individual chamber with free access to water and CFD or HFD. Respiratory quotient (RQ), energy expenditure (cal/min/kg), fat oxidation (mg/min/kg), and mice activity (counts/min) were measured every 5 min during 48 h.

In vitro insulin secretion. For measurement of glucose-stimulated insulin secretion (GSIS) *in vitro*, islets from mice on CFD and HFD were isolated using collagenase digestion method. In brief, mice were sacrificed by cervical dislocation; 0.5 mg/ml collagenase dissolved in Hanks balanced salt solution (HBSS) 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 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 20 mM $NaHCO_3$) at 37°C during 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 light microscope. For GSIS assessment, three batches of different