

Mechanisms of fat-induced gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide secretion from K cells

Shunsuke Yamane¹, Norio Harada¹, Nobuya Inagaki^{1*}

Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Keywords

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*Correspondence

Nobuya Inagaki
 Tel.: +81-75-751-3562
 Fax: +81-75-771-6601
 E-mail address: inagaki@metab.kuhp.
 kyoto-u.ac.jp

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ABSTRACT

Gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) is one of the incretins, which are gastrointestinal hormones released in response to nutrient ingestion and potentiate glucose-stimulated insulin secretion. Single fat ingestion stimulates GIP secretion from enteroendocrine K cells; chronic high-fat diet (HFD) loading enhances GIP secretion and induces obesity in mice in a GIP-dependent manner. However, the mechanisms of GIP secretion from K cells in response to fat ingestion and GIP hypersecretion in HFD-induced obesity are not well understood. We generated GIP-green fluorescent protein knock-in (GIP^{gfp/+}) mice, in which K cells are labeled by enhanced GIP-green fluorescent protein. Microarray analysis of isolated K cells from GIP^{gfp/+} mice showed that both fatty acid-binding protein 5 and G protein-coupled receptor 120 are highly expressed in K cells. Single oral administration of fat resulted in significant reduction of GIP secretion in both fatty acid-binding protein 5- and G protein-coupled receptor 120-deficient mice, showing that fatty acid-binding protein 5 and G protein-coupled receptor 120 are involved in acute fat-induced GIP secretion. Furthermore, the transcriptional factor, regulatory factor X6 (Rfx6), is highly expressed in K cells. *In vitro* experiments using the mouse enteroendocrine cell line, STC-1, showed that GIP messenger ribonucleic acid levels are upregulated by Rfx6. Expression levels of Rfx6 messenger ribonucleic acid as well as that of GIP messenger ribonucleic acid were augmented in the K cells of HFD-induced obese mice, in which GIP content in the small intestine is increased compared with that in lean mice fed a control diet. These results suggest that Rfx6 is involved in hypersecretion of GIP in HFD-induced obese conditions by increasing GIP gene expression.

GIP AND OBESITY

Obesity is recognized as a worldwide problem, especially for developing insulin resistance and increasing the risk of type 2 diabetes¹. The average body mass index (BMI) of Japanese diabetic patients has been increasing in recent years, in parallel with an increase in dietary fat intake. Preventing diet-induced obesity has become an urgent challenge for society as a whole.

Gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretins, peptide hormones released from the gastrointestinal

tract into circulation in response to nutrient ingestion that potentiate glucose-stimulated insulin secretion^{2–4}. GIP is secreted from K cells located in the upper small intestine; GLP-1 is secreted from L cells located in the lower small intestine and colon. Dietary lipid is an especially strong stimulant of GIP secretion. Total plasma GIP levels in wild-type (WT) mice after oral lard administration are much higher than that after oral glucose administration⁵. The peak value of plasma GIP in response to a high-fat meal (450 kcal containing 33.3% fat) is threefold higher than that by 75 g oral glucose tolerance test in human subjects, suggesting that the fat content in a mixed meal strongly stimulates GIP secretion⁶.

GIP is considered to increase the volume of adipose tissue by two major pathways: directly by binding to the GIP receptor located on the adipocytes^{7,8} and indirectly, by accelerating fat

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deposition and expansion of fat depots by increasing insulin secretion from pancreatic β -cells⁹. Studies of GIP receptor knockout mice show GIP to be an obesity-promoting factor in high-fat diet (HFD) conditions¹⁰, and show deletion of GIP receptor signaling to cause resistance to diet-induced obesity¹¹. Additionally, we reported that partial reduction of GIP alleviates obesity and lessens the degree of insulin resistance without exacerbating glucose tolerance under HFD conditions¹². Increased plasma GIP levels in obesity have been shown in several studies^{13–16}. We previously reported that the plasma GIP level after glucose loading is positively correlated with body mass index in healthy subjects¹⁷. Furthermore, a report showing that healthy human subjects given high fat food for 2 weeks show increased plasma GIP levels without developing obesity, suggesting that GIP hypersecretion precedes obesity¹⁸.

These findings suggest that there are both acute mechanisms of GIP secretion in response to a single administration of fat and chronic mechanisms for hyperproduction of GIP under HFD feeding. However, the precise mechanisms of GIP secretion and GIP production have remained unclear, mainly because of inability to isolate GIP-producing K cells from intestinal epithelium. Recently, we generated GIP-GFP knock-in mice, in which K cells are labeled by enhanced green fluorescent protein, and have succeeded in isolating K cells using a

flow cytometry technique. On the basis of microarray analysis of K cells isolated from GIP-GFP knock-in heterozygous (GIP^{gf/+}) mice, we showed that some factors highly expressed in K cells are involved in fat-induced GIP secretion and GIP hypersecretion in diet-induced obesity.

FATTY ACID-BINDING PROTEIN 5

Fatty acid-binding protein 5 (FABP5) is a 15 kDa cytosolic protein with a high affinity to long chain fatty acids, which has been known as an intracellular chaperon transporting long chain fatty acids into various organelles. Using microarray analysis, we showed that FABP5 is expressed in murine K cells, and investigated the physiological function of FABP5 in K cells⁵. Immunostaining of intestinal mucosa showed that GIP-positive cells were totally merged with FABP5-positive cells, and that 90% of FABP5-positive cells were merged with GIP-positive cells. To evaluate the acute GIP secretory response in FABP5 knockout (FABP5^{-/-}) mice, lard (10 mL/kg) and glucose (2 mg/kg) were injected orally, and plasma glucose and serum levels of GIP, GLP-1 and insulin were measured. Plasma glucose, insulin and GLP-1 levels after both glucose and lard administration were similar in FABP5^{+/+} mice and FABP5^{-/-} mice (Figure 1a–c,e–g). Plasma GIP levels after lard injection were significantly lower in FABP5^{-/-} mice compared with those

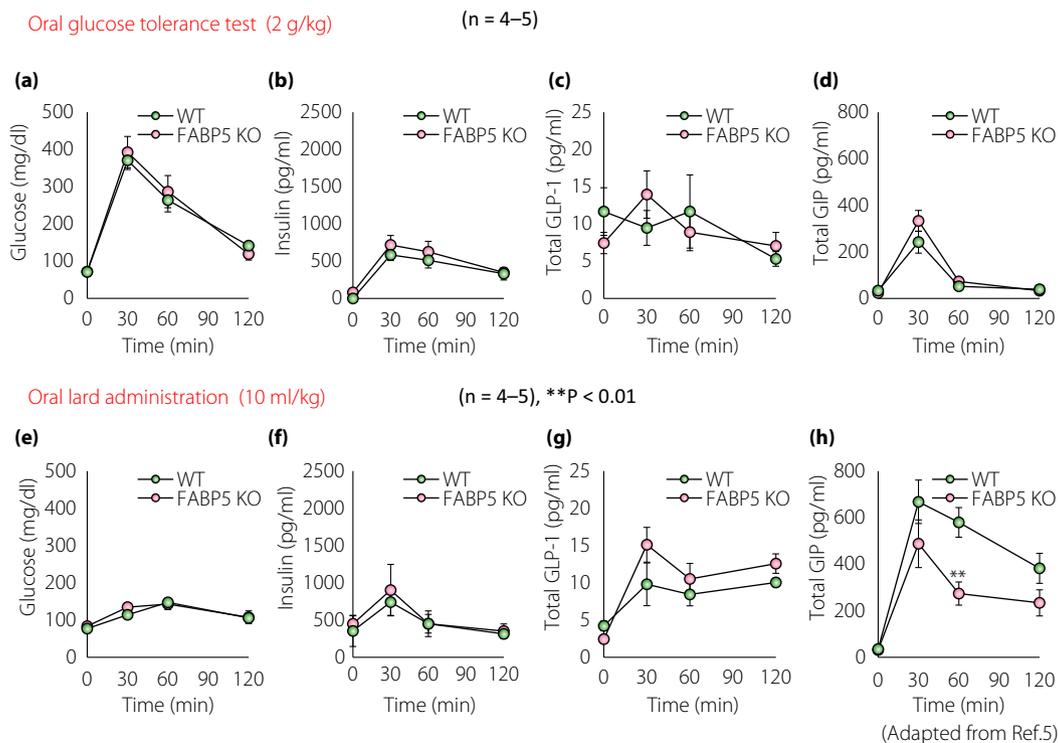


Figure 1 | Oral glucose and lard oil tolerance tests to fatty acid-binding protein 5 (FABP5) knockout mice. Concentrations of (a,e) blood glucose, (b, f) serum insulin, (c,g) plasma total glucagon-like peptide-1 (GLP-1) and (d,h) plasma total gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) after oral administration of (a–d) 2 g/kg glucose and (e–g) 10 mL/kg lard. ****P < 0.01** between wild-type mice (WT; n = 4; green circle) and FABP5 knockout mice (FABP5 KO; n = 5; orange circle) mice.

in FABP5^{+/+} mice (Figure 1h), but there were no significant differences in the results of oral glucose tolerance test (Figure 1d). Given that FABPs are known to function as an intracellular lipid chaperone, we speculated that there would be a mechanism by which fatty-acid permeates the cell membrane of K cells for FABP5-associated GIP secretion in response to fat ingestion. To examine the significance of micelle-aided incorporation of long chain fatty acids into K cells in the presence of bile, as is the case with absorptive epithelial cells in the small intestine, we evaluated GIP secretion after glucose or lard administration in mice subjected to common bile duct ligation (BDL), in comparison with mice subjected to sham operation. Although GIP secretion after glucose injection remained unaffected by BDL, the secretion after lard injection was seriously diminished in BDL mice. Dissociation of the effect of BDL on GIP secretion after glucose and lard administration suggests that bile is the crucial factor in GIP secretion in response to fat, but not to glucose. As an *ex vivo* experiment, the upper half portion of the small intestine was harvested from FABP5^{+/+} and FABP5^{-/-} mice, shredded into small pieces, and incubated in conditioned media as follows: 5.5 mmol/L glucose Dulbecco's modified Eagle medium as a control, 100 μmol/L oleic acid, 4 v/v% of bile and 100 μmol/L oleic acid plus 4 v/v% of bile. We found no GIP release after 15 min incubation with bile, and a very small increase in GIP concentration in the media after the incubation with oleic acid. By contrast, there were marked increases of GIP secretion from the samples incubated with oleic acid plus bile, and the FABP5^{-/-} samples showed a 3.4-fold decrease compared with that in FABP5^{+/+} samples. To assess potential effects of FABP5-deficiency on bodyweight and composition, mice were fed HFD for 10 weeks, and whole-body computed tomography scans of HFD-fed FABP5^{+/+}, FABP5^{-/-}, GIP-GFP knock-in homozygous (GIP^{gfp/gfp}-FABP5^{+/+} and GIP^{gfp/gfp}-FABP5^{-/-} mice were compared. Under HFD feeding conditions, FABP5^{-/-} mice showed significantly decreased bodyweight gain compared with FABP5^{+/+} mice, but there was no significant difference in bodyweight between GIP^{gfp/gfp}-FABP5^{+/+} and GIP^{gfp/gfp}-FABP5^{-/-} mice, in which GIP expression is genetically deleted. Whole-body computed tomography scan showed that body fat mass was significantly reduced in FABP5^{-/-} mice compared with that in FABP5^{+/+} mice, and that body fat mass in GIP^{gfp/gfp}-FABP5^{+/+} and GIP^{gfp/gfp}-FABP5^{-/-} mice was comparable. These results show that FABP5 is involved in fatty acid-induced acute GIP secretion, and that it contributes to the development of HFD-induced obesity in a GIP-dependent manner.

G PROTEIN-COUPLED RECEPTOR 120

Receptors for long-chain fatty acids (G protein-coupled receptor [GPR]40 and GPR120)^{19,20}, short-chain fatty acids (GPR41 and GPR43)²¹ and oleoylethanolamide (GPR119)²² are known to be involved in GLP-1 secretion. However, the role of fatty acid-sensing G protein-coupled receptors (GPCRs), except for GPR40 and GPR119, in GIP secretion from K cells remains unclear^{19,22}.

To evaluate the expression levels of fatty acid-sensing GPCRs in K cells, we carried out reverse transcription polymerase chain reactions using isolated K cells from GIP-GFP knock-in mice²³. Although GPR119 showed a tendency toward abundance in GFP-positive K cells of the lower small intestine, the difference between GFP-positive and GFP-negative cells was not significant. GPR40 and GPR43 were highly expressed in K cells of the lower small intestine. In contrast, GPR120 was highly expressed in K cells of the upper small intestine and not of the lower small intestine. We have found that not only K cell number, but also GIP content and GIP messenger ribonucleic acid (mRNA) expression in K cells are greater in the upper small intestine compared with those in the lower small intestine²³, suggesting that K cells in the upper small intestine contribute more to nutrient-induced GIP secretion than K cells of the lower small intestine. In such a context, it might be assumed that GPR120, which is highly expressed in K cells of the upper small intestine, is crucial for fatty acid-induced GIP secretion.

To ascertain the role of GPR120 in GIP secretion, we measured plasma GIP levels after oral lard oil administration in WT mice and GPR120 knockout (GPR120^{-/-}) mice²³. Glucose-induced GIP secretion was similar between WT and GPR120^{-/-} mice (Figure 2a,b). In contrast, GIP secretion induced by lard oil in GPR120^{-/-} mice was significantly reduced to 25% of that in WT mice (Figure 2c,d). Furthermore, we examined the effect of grifolic acid methyl ether, which competitively inhibits long-chain fatty acid-induced activation of GPR120 signaling²⁴, on GIP secretion. Treatment of mice with grifolic acid methyl ether resulted in decreased lard oil-induced GIP secretion, but not in glucose-induced GIP secretion. These results suggest that fatty acids derived from lard oil stimulate GIP secretion through GPR120 signaling.

REGULATORY FACTOR X6

Several studies have reported that GIP secretion is increased in obesity¹³⁻¹⁶. However, the mechanisms involved in GIP hypersecretion from K cells in obesity remain unclear, mainly because of difficulties in separating these cells from other intestinal epithelial cells *in vivo*. It has been shown that pancreatic and duodenal homeobox 1 (Pdx1), which is known to be an important transcription factor in pancreatic development and pancreatic β-cell maturation²⁵, has a critical role in GIP production in K cells^{26,27}. However, the contribution of Pdx1 and other transcription factors in obesity-associated GIP hypersecretion has not been confirmed. Based on microarray analysis data, we showed that mRNA of regulatory factor X6 (Rfx6) is highly expressed in K cells²⁸. Immunohistochemistry of the small intestine from GIP-GFP knock-in mice confirmed that Rfx6-expressing cells correspond to GFP-expressing cells, showing that Rfx6 is highly expressed in K cells in the murine small intestine.

The *Rfx* gene family of transcription factors was first detected in mammals as regulatory factors that bind to the promoter

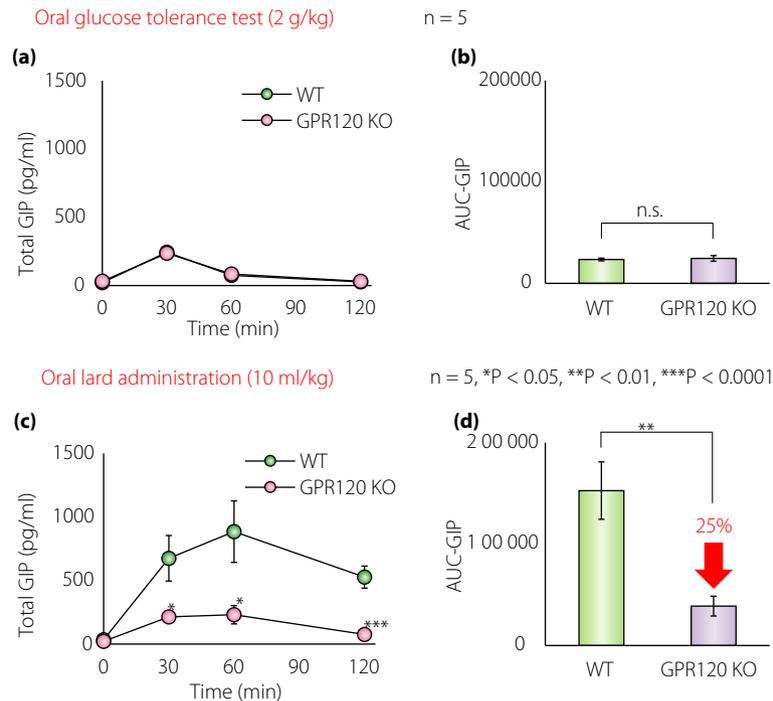


Figure 2 | Gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) secretion in G protein-coupled receptor 120 (GPR120) knockout mice. Concentrations of total plasma GIP after oral administration of (a) 2 g/kg glucose and (c) 10 mL/kg lard. Area under the curve (AUC) of GIP concentrations after oral administration of (b) glucose and (d) lard. GPR120 KO; WT, * $P < 0.05$, ** $P < 0.01$ between wild-type mice (WT; $n = 5$; green circle/green bar) and GPR120 knockout mice (GPR120 KO; $n = 5$; orange circle/orange bar). NS, not significant.

regions of major histocompatibility complex class II genes²⁹; seven types of Rfx (Rfx1–7) have so far been identified. All Rfx transcription factors have a winged helix deoxyribonucleic acid binding domain. Rfx1–4 and -6 have a dimerization domain^{30,31}, and Rfx6 forms homodimers or heterodimers with Rfx2 or Rfx3^{32,33}. Rfx6 was initially isolated from human genome sequences in 2008³⁰. Serial Analysis of Gene Expression (SAGE) frequency data showed high expression of Rfx6 mRNA in the pancreas, liver and heart, and reverse transcription polymerase chain reaction analysis showed high expression of Rfx6 mRNA in the human pancreas and intestine³⁴. Rfx6-deficient mice were previously generated, and none of the endocrine cells, excluding pancreatic polypeptide-expressing cells, were detected in the islets of these mice³⁴. These results suggest that Rfx6 plays a critical role in generating the endocrine cells in islets, but it has been unknown whether Rfx6 is associated with generation of enteroendocrine cells, such as K cells.

To examine the involvement of Rfx6 in GIP gene expression, we evaluated GIP mRNA expression and content under the inhibition of Rfx6 expression *in vitro*. By treatment with Rfx6 small interfering RNA, mRNA expression and cellular content of GIP were significantly decreased in mouse enteroendocrine cell line STC-1 cells. We assessed the interaction of the Rfx6 and the GIP gene by one-hybrid assay. Rfx6 effectively bound to a fragment of the GIP promoter (5216–6512 bp upstream of the GIP promoter). In the luciferase promoter assay, GIP pro-

motor activity of the fragments containing 5216–6512 bp upstream of the GIP promoter was high. These results suggest that Rfx6 binds to the region 5216–6512 bp upstream of the GIP promoter, which regulates GIP promoter activity. Furthermore, we confirmed that GIP mRNA expression levels were significantly increased in Rfx6-overexpressing STC-1 cells, showing that Rfx6 expressed by K cells plays an important role in GIP gene expression.

To investigate the mechanisms of GIP hypersecretion in HFD-induced obesity *in vivo*, GIP-GFP heterozygous mice were fed a control-fat diet (CFD) or HFD, and K cells were isolated for further evaluation. From 1 week after starting these diets onward, the bodyweight of the HFD group remained significantly higher compared with that of the CFD group. After CFD or HFD feeding for 8 weeks, oral glucose tolerance tests were carried out. GIP secretion (area under the curve-GIP) of the HFD group was increased by approximately 1.5-fold compared with that of the CFD group. These results show that HFD feeding increases GIP secretion and induces obesity in GIP-GFP heterozygous mice, even though they have only one normal GIP gene, indicating that these mice represent a useful model for analysis of the mechanisms involved in the augmentation of GIP secretion in HFD-induced obesity.

To determine whether GIP hypersecretion is caused by an increased number of K cells in HFD-fed GIP-GFP heterozy-

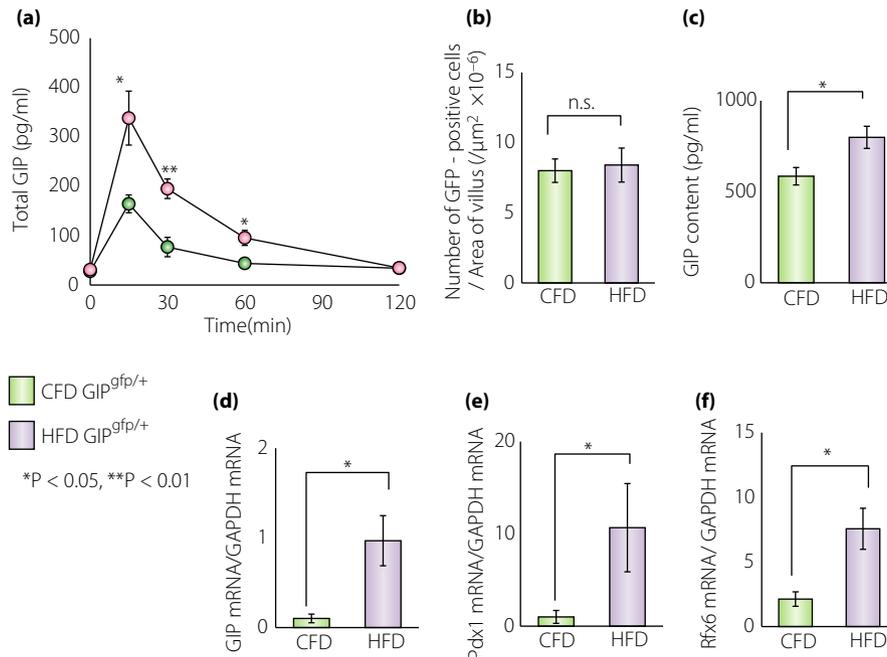


Figure 3 | Increased expression levels of pancreatic and duodenal homeobox 1 (Pdx1) and regulatory factor X6 (Rfx6) messenger ribonucleic acid (mRNA) in K cells after high-fat diet (HFD) feeding. (a) Concentrations of plasma total gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) after oral administration of 2 g/kg glucose in control-fat diet (CFD)-fed GIP-green fluorescent protein knock-in (GIP^{gf/+}) mice (n = 5; green circle) and HFD-fed GIP^{gf/+} mice (n = 5; orange circle). *P < 0.05, **P < 0.01. (b) Number of K cells in the upper small intestine (n = 5). (c) GIP content in the upper small intestine (n = 5–7). (d) Expression of GIP mRNA in K cells (n = 8–10). (e) Expression of Pdx1 mRNA in K cells (n = 8–10). (f) Expression of Rfx6 mRNA in K cells (n = 8–10). GFP-positive cells from CFD-fed GIP^{gf/+} mice (green bar), from HFD-fed GIP^{gf/+} mice (orange bar). *P < 0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. NS, not significant.

gous mice, the number of K cells in the upper small intestine of CFD-fed mice and HFD-fed mice were estimated and compared. We could not detect an increase of K cell number in the duodenum or upper small intestine of HFD-fed GIP-GFP heterozygous mice by immunohistochemistry and flow cytometry analysis. In addition, in K cells purified by using flow cytometry, the expression levels of GIP mRNA were almost 10-fold higher in the HFD group than those in the CFD group. These results show that GIP hypersecretion under HFD-induced obesity is not due to an increase in K cell number, but to an increase of GIP mRNA expression and content in K cells. Furthermore, the expression levels of Rfx6 and Pdx1 mRNA were significantly increased in K cells of HFD-induced obese mice compared with those of CFD-fed lean mice (Figure 3). In previous studies, it has been reported that Pdx1 binds 150 bp upstream of the GIP promoter and activates the GIP promoter in STC-1 cells, and that Pdx1 expression is essential for producing GIP in K cells^{26,27}. Thus, an increase in Rfx6 and Pdx1 expressions in K cells of HFD-induced obese mice is consistent with the *in vitro* data showing that Rfx6 and Pdx1 are involved in GIP gene expression.

In summary, we found that transcription factor Rfx6 is highly expressed in K cells, and is involved in the regulation of GIP expression. We also showed that expression of Rfx6 and

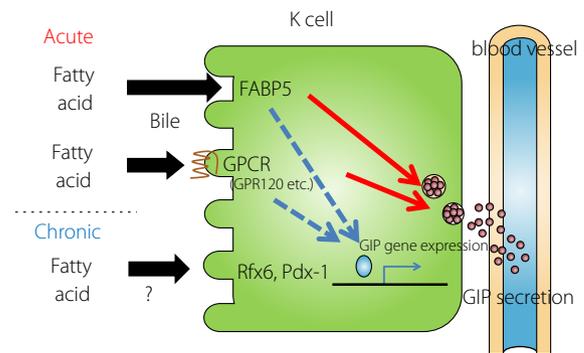


Figure 4 | The mechanisms of fat-induced gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) secretion and increased GIP synthesis in response to after high-fat diet (HFD) feeding. Fatty acid-binding protein 5 (FABP5) and G protein-coupled receptor 120 (GPR120) play crucial role in acute fat-induced GIP secretion and regulatory factor X6 (Rfx6) is involved in hypersecretion of GIP in HFD-induced obese conditions by increasing GIP gene expression. GPCR, G protein-coupled receptor; Pdx-1, pancreatic and duodenal homeobox 1.

Pdx1 is upregulated in the K cells of HFD-induced obese mice, which suggests that induction of Rfx6 as well as Pdx1 plays a critical role in GIP hypersecretion in HFD-induced obesity.

CONCLUSION

Gene analysis of K cells isolated from GIP-GFP mice enabled us to identify candidate genes that contribute to acute and chronic mechanisms stimulating GIP secretion in response to fat ingestion: FABP5 and GPR120 play crucial roles in acute fat-induced GIP secretion, and Rfx6 is involved in hypersecretion of GIP in HFD-induced obese conditions by increasing GIP gene expression (Figure 4). Although further, detailed analyses are required to clarify the intracellular signaling of GIP secretion and synthesis in response to nutrient ingestion, regulation of GIP secretion could provide a novel therapeutic approach to prevent obesity, insulin resistance and subsequent type 2 diabetes.

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DISCLOSURE

SY and NH have no conflict of interest. NI served as a medical advisor for Takeda, Taisho Pharmaceutical, GlaxoSmithKline and Mitsubishi Tanabe Pharma, and lectured for MSD, Sanofi, Novartis Pharma, Dainippon Sumitomo Pharma, Kyowa Kirin and Mitsubishi Tanabe Pharma, and received payment for his services. NI also received a clinical commissioned/joint research grant from MSD, Eli Lilly Japan, Shiratori Pharmaceutical, Roche Diagnostics and the Japan Diabetes Foundation, and also received a scholarship grant from MSD, Japan Tobacco Inc., Nippon Boehringer Ingelheim, Takeda, Dainippon Sumitomo Pharma, Astellas Pharma, Daiichi-Sankyo and Mitsubishi Tanabe Pharma.

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