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ORIGINAL ARTICLE

Free fatty acid receptors, G protein-coupled receptor 120 and G protein-coupled receptor 40, are essential for oil-induced gastric inhibitory polypeptide secretion

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ABSTRACT

Aims/Introduction: Incretin hormone glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) plays a key role in high-fat diet-induced obesity and insulin resistance. GIP is strongly secreted from enteroendocrine K cells by oil ingestion. G protein-coupled receptor (GPR)120 and GPR40 are two major receptors for long chain fatty acids, and are expressed in enteroendocrine K cells. In the present study, we investigated the effect of the two receptors on oil-induced GIP secretion using GPR120- and GPR40-double knockout (DKO) mice.

Materials and Methods: Global knockout mice of GPR120 and GPR40 were crossbred to generate DKO mice. Oral glucose tolerance test and oral corn oil tolerance test were carried out. For analysis of the number of K cells and gene expression in K cells, DKO mice were crossbred with GIP-green fluorescent protein knock-in mice in which visualization and isolation of K cells can be achieved.

Results: Double knockout mice showed normal glucose-induced GIP secretion, but no GIP secretion by oil. We then investigated the number of K cells and gene characteristics in K cells isolated from GIP-green fluorescent protein knock-in mice. Deficiency of both receptors did not affect the number of K cells in the small intestine or expression of GIP messenger ribonucleic acid in K cells. Furthermore, there was no significant difference in the expression of the genes associated with lipid absorption or GIP secretion in K cells between wild-type and DKO mice.

Conclusions: Oil-induced GIP secretion is triggered by the two major fatty acid receptors, GPR120 and GPR40, without changing K-cell number or K-cell characteristics.

INTRODUCTION

The explosive increase in the number of obese individuals today results from energy-balance dysregulation derived from environmental factors, consumption of high caloric food and less time spent in physical activities[1]. As obesity is closely linked with the leading causes of death and disability, such as cancer and diabetes, it is important to establish means to prevent and treat obesity.

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The intestine is the main organ for digestion of nutrients, and is the largest endocrine system in the mammalian body. In response to food intake, numerous hormones are released from the intestine to orchestrate nutrient absorption, energy accumulation and glucose homeostasis. Incretins are released from K cells and L cells, and potentiate glucose-dependent insulin secretion from pancreatic β-cells. Glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the two major incretins. Enteroendocrine K cells are present mainly in the upper small

intestine, and secrete GIP[2]; enteroendocrine L cells are present in the small intestine and colon, and secrete GLP-1[3].

In addition to its role as an incretin, GIP is also known to play a key role in high-fat diet (HFD)-induced obesity. GIP receptor-knockout mice and GIP-knockout mice are both resistant to HFD-induced obesity and insulin resistance under a HFD condition[4,5]. Thus, GIP has potential as a therapeutic target in the treatment of HFD-induced obesity and insulin resistance[6,7]. As lipids are the strongest secretagogue of GIP, the lipid-sensing system in K cells has drawn much attention. GPR120 and GPR40 are both identified as G protein-coupled receptors (GPCR) for long-chain fatty acids[8,9]. As GPR120 and GPR40 are expressed on enteroendocrine cells, including K cells, in a highly selective manner[2,10], they might well be involved in oil-induced GIP secretion as the lipid sensors in K cells. In this decade, both GPR120 and GPR40 global knockout mice have been reported to show impaired GIP secretion[2,11]. We have shown that the extent of the contribution of GPR40 in GIP secretion is greater compared with that of GPR120 by direct comparison of GPR120- and GPR40-knockout mice[12]. However, the effect of these two major receptors on GIP secretion in response to lipids remains unclear. Furthermore, there are questions regarding K-cell number in the small intestine and gene expression in K cells under deficiency of both receptors. In the present study, we generated GPR120- and GPR40-double knockout (DKO) mice, and investigated the effect of the receptors on GIP secretion and K-cell characteristics.

METHODS

Animals

Global knockout GPR120[13] and GPR40[14] mice were crossbred to generate DKO mice. For analysis of K-cell number and gene expression in K cells, DKO mice were crossbred with GIP-green fluorescent protein (GFP) knock-in (GIP-GFP) mice in which visualization and isolation of K cells can be achieved[15]. All experiments in the present study used age-matched (aged 8–16 weeks old) male littermates maintained on the C57/BL6J background. All mice were housed in a temperature-controlled environment under conditions of 14:10-h light/dark cycle with free access to water and chow unless otherwise stated. Animals were maintained according to protocols approved by the Animal Care Committee of Kyoto University (MedKyo 16548).

Oral glucose and corn oil tolerance test

Oral glucose tolerance tests (OGTTs) and oral corn oil tolerance tests (OCTTs) were carried out after a 16-h fasting period. Mice were administered glucose by gavage (6 g/kg bodyweight for GLP-1 measurement, 2 g/kg bodyweight for others) for OGTTs, and corn oil (10 mL/kg body weight) for OCTTs. Then, 45-μL blood samples were collected via tail vein at 0 (15 for OGTTs), 30, 60 and 120 min after oral glucose or oil administration, and blood glucose, plasma insulin, plasma total GIP and plasma total GLP-1 levels were measured. Intraperitoneal corn oil tolerance tests were carried out after a 16-h fasting period. Corn oil (10 mL/kg bodyweight) was intraperitoneally administered, and 45-μL blood samples were collected via tail vein at 0, 30, 60 and 90 min to measure total GIP levels. Blood glucose levels were measured by the glucose oxidase method (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Plasma insulin, triglyceride, total GIP, total GLP-1 and peptide YY levels were measured by insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Shibukawa, Japan), triglyceride assay kit (Wako, Osaka, Japan), total GIP ELISA kit (Merck Millipore, Billerica, MA, USA), total GLP-1 ELISA kit (Meso Scale Discovery, Rockville, MD, USA) and peptide YY ELISA kit (Yanaihara Institute Inc., Fujimi, Japan), respectively. After anesthesia with isoflurane inhalation, bile in the gallbladder was collected with a 29-G syringe at 30 min after oral corn oil administration, and bile volume was measured.

Isolation of K cells from mouse upper intestinal epithelium

The upper half portion of the small intestine, in which K cells in the gastrointestinal tract are found most abundantly, was examined in the present study. K cells were collected from GIP-GFP heterozygous mice and DKO GIP-GFP heterozygous mice, as previously described[12]. GFP-positive cells were counted and collected as K cells using a BD fluorescence activated cell sorter Aria flow cytometer (Becton Dickinson, San Jose, CA, USA).

Ribonucleic acid isolation and real-time polymerase chain reaction

A total of 2000 GFP-positive cells (K cells) were collected into extraction buffer of the Picopure RNA isolation kit (Applied Biosystems, Alameda, CA, USA). Total ribonucleic acid (RNA) was extracted from the cells and was treated with deoxyribonuclease (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed using reverse transcription (Invitrogen, Carlsbad, CA, USA) with an oligo-deoxythymidine primer (Invitrogen) to prepare complementary deoxyribonucleic acid. The quantitative polymerase chain reaction was carried out using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All results are presented using the ΔCt method normalized to a housekeeping gene (β-actin). Primer sequences are shown in Table S1.

Statistical analysis

The results are shown as the mean ± standard error of the mean. Statistical significance was determined using Student's *t*-test. *P*-values <0.05 were considered statistically significant.

RESULTS

DKO mice show severely impaired oil-induced GIP secretion

After oral glucose administration, wild-type (WT) and DKO mice showed similar blood glucose (Figure 1a) and plasma insulin (Figure 1c) levels. After oral corn oil administration, blood glucose levels were slightly higher in DKO mice than

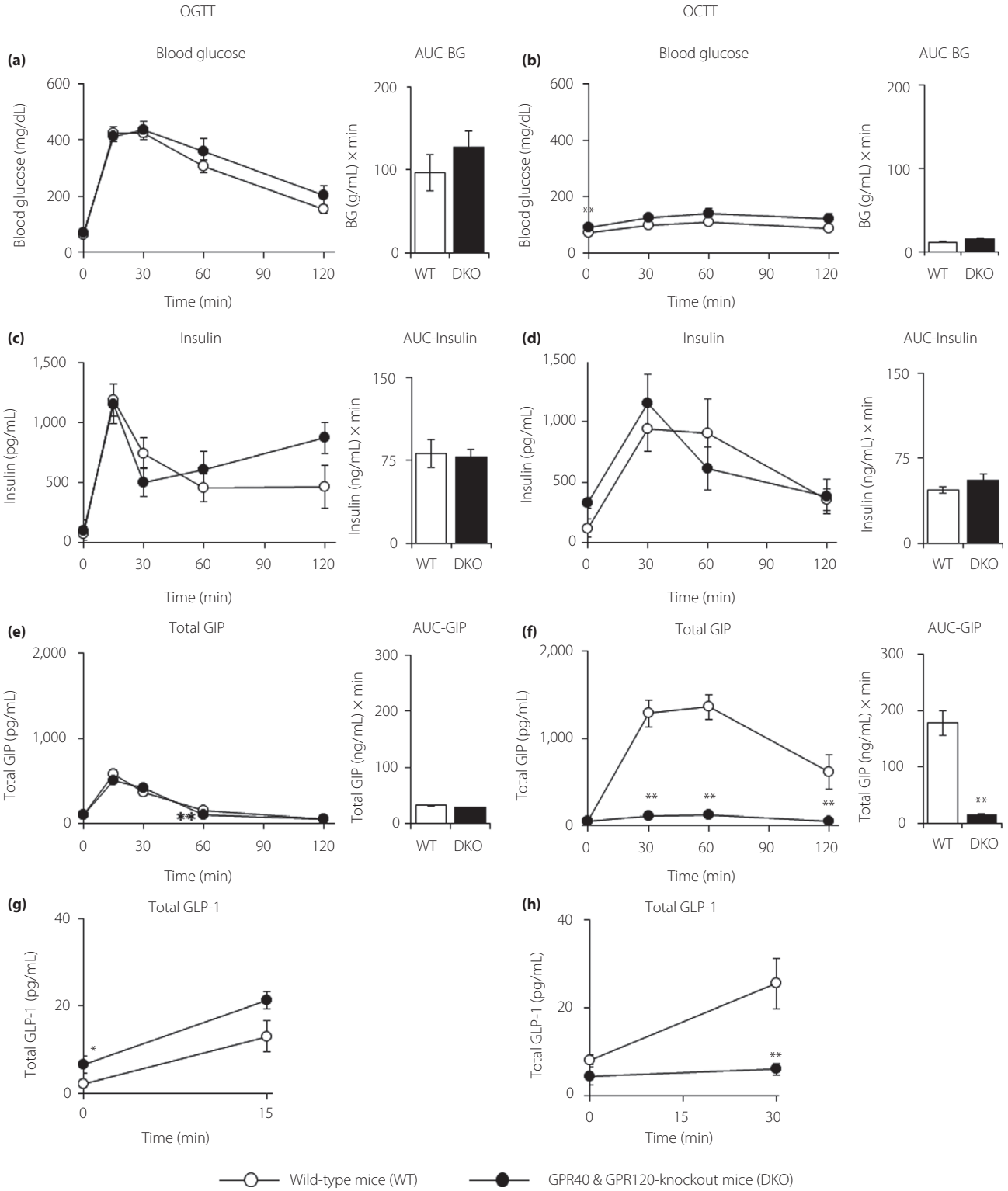

Figure 1 | Blood glucose, insulin and incretin levels during oral glucose tolerance tests (OGTTs) and oral corn oil tolerance tests (OCTTs). (a,b) Blood glucose (BG), (c,d) insulin, (e,f) total gastric inhibitory polypeptide (GIP) and (g,h) total glucagon-like peptide-1 (GLP-1) levels during OGTTs (2 g/kg glucose [blood glucose, insulin and total GIP] or 6 g/kg glucose [total GLP-1]) and OCTTs (10 mL/kg corn oil; $n = 4–6$). Wild-type (WT) mice are represented by white circles and bars. Double knockout (DKO) mice are represented by black circles and bars. *$P < 0.05$, **$P < 0.01$ versus WT mice. AUC, area under the curve.

those in WT mice, but the difference was not statistically significant (Figure 1b). Plasma insulin levels during OCTTs did not differ between the two groups (Figure 1d).

DKO mice and WT mice secreted GIP equally during OGTT (Figure 1e). In contrast, DKO mice did not show elevated plasma GIP levels, except at 60 min after corn oil administration (Figure 1f). DKO mice showed a 92% reduction of the area under the curve of GIP. Thus, the impairment of GIP secretion in DKO mice after oil administration is severe. We measured plasma triglyceride levels at 30 min in OCTTs, and found no significant difference between WT and DKO mice (data not shown).

We measured GLP-1 levels at 15 and 30 min during OGTTs and OCTTs, respectively, as we observed peaks of GLP-1 levels at 15 min after glucose load and at 30 min after oil administration[12]. Whereas GLP-1 secretion was comparable during OGTTs (Figure 1g), DKO mice did not show significant elevations of GLP-1 levels during OCTTs (Figure 1h). Peptide YY levels in OGTTs and OCTTs were not significantly different between WT and DKO mice (data not shown).

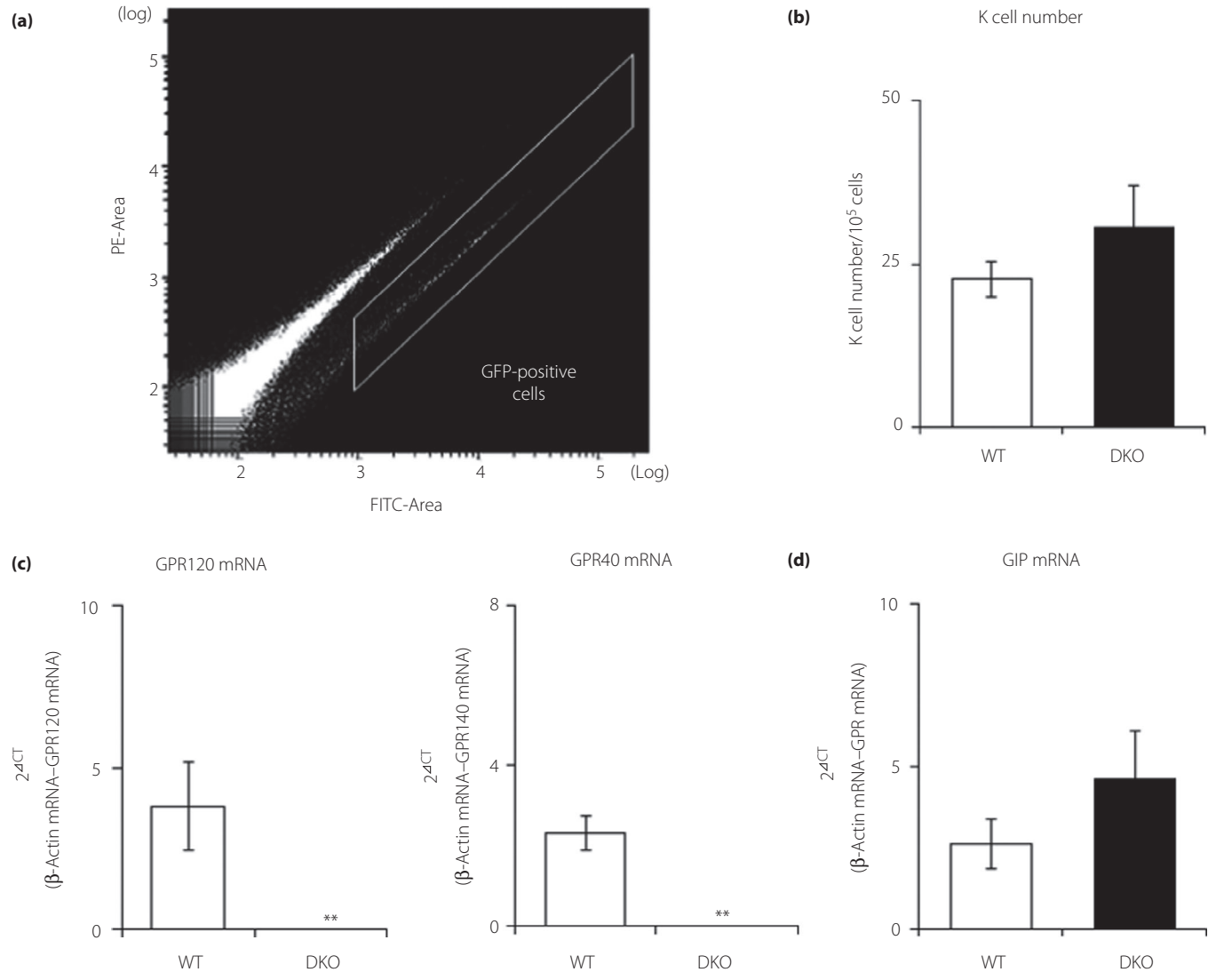

Figure 2 | K-cell number and gastric inhibitory polypeptide (GIP) expression in K cells. (a) FACS gating for green fluorescent protein (GFP) expression in single dispersed intestinal epithelial cells. (b) The number of GFP-positive cells (K cells) in the small intestine counted by flow cytometer (n = 5). The expression levels of (c) G protein-coupled receptor (GPR)120 messenger ribonucleic acid (mRNA), GPR40 mRNA and (d) GIP mRNA in the K cells (n = 5). Wild-type (WT) and double knockout (DKO) mice are represented by white and black bars, respectively. **P < 0.01 versus WT mice. FITC, fluorescein isothiocyanate.

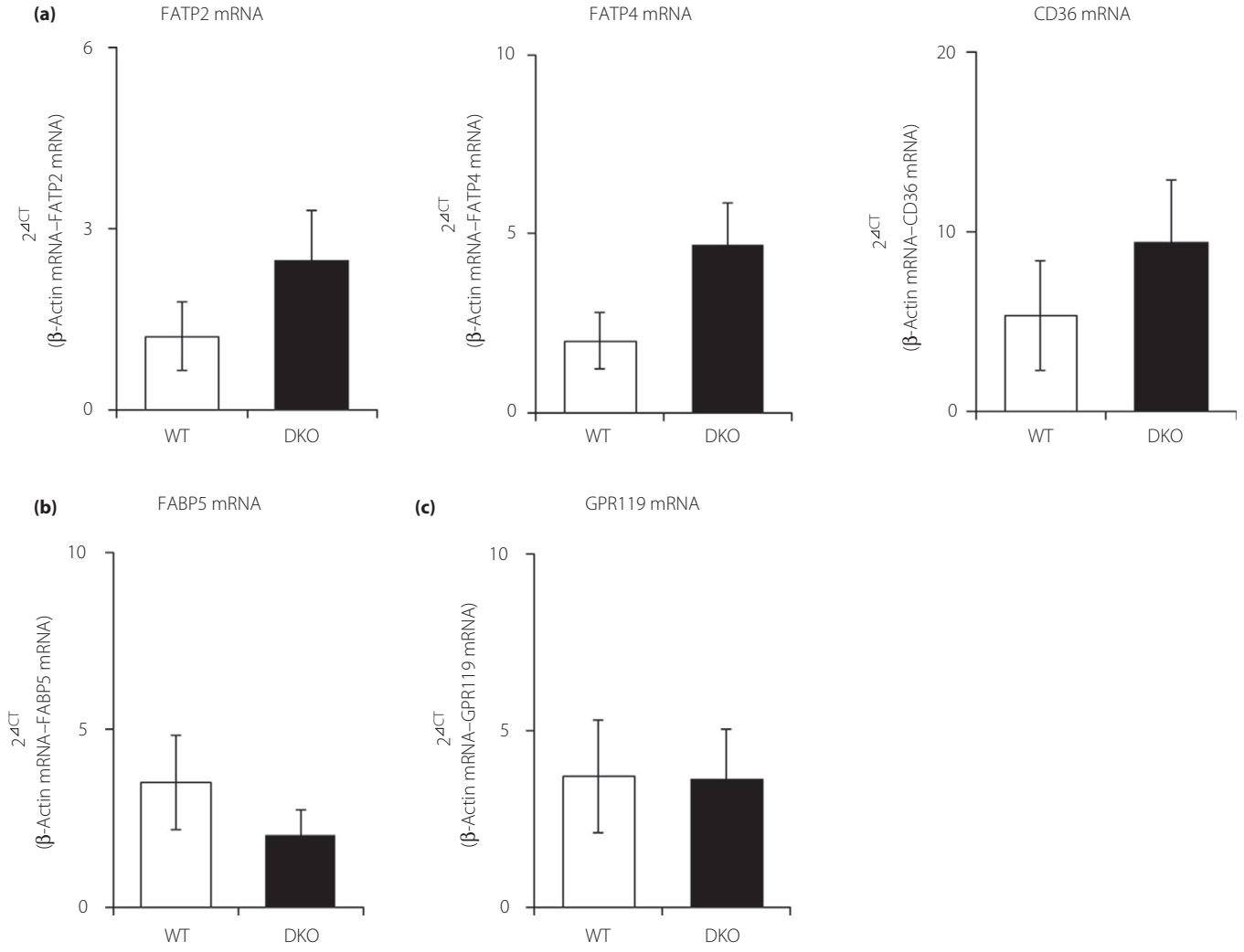

Figure 3 | Expression of fatty acid-associated genes in K cells. The messenger ribonucleic acid (mRNA) expression levels of (a) fatty acid transport protein (FATP)2, FATP4, CD36, (b) FABP5 and (c) G protein-coupled receptor (GPR)119 in K cells (n = 5). Wild-type (WT) and double knockout (DKO) mice are represented by white and black bars, respectively.

We measured the bodyweight of WT mice and DKO mice from 8 to 18 weeks old under a normal diet-fed condition (11% fat), and found no statistical difference between them (data not shown).

Effect of GPR120 and GPR40 double deficiency on K-cell number and GIP expression in K cells

GFP-positive cells were purified as K cells from the upper small intestine by flow cytometer (Figure 2a). The number of K cells in DKO mice was comparable to that of WT mice (Figure 2b). We confirmed that GPR120 and GPR40 messenger RNA (mRNA) expressions were completely diminished in the K cells of DKO mice (Figure 2c). There was no significant difference in GIP mRNA expressions in K cells between WT and DKO mice (Figure 2d).

Gene expression of fatty acid transporters and GPR119 in the K cells of DKO mice

To investigate the cause of severe impairment of oil-induced GIP secretion in DKO mice, we evaluated the mRNA expressions of CD36 (fatty acid translocator [FAT]) and fatty acid transporters, such as fatty acid transport protein (FATP) 1–5. FATP1, 3 and 5 mRNA expression levels were under the detectable range in K cells (data not shown). The expression levels of CD36, FATP2 and FATP4 mRNA did not differ between the two groups; their expression levels were all within the measureable range in K cells (Figure 3a). Fatty acid-binding protein (FABP) is known as an intracellular chaperone that transports long-chain fatty acids into various organelles[16]. We previously reported that FABP5 is highly expressed in K cells, and plays a crucial role in GIP secretion after fat

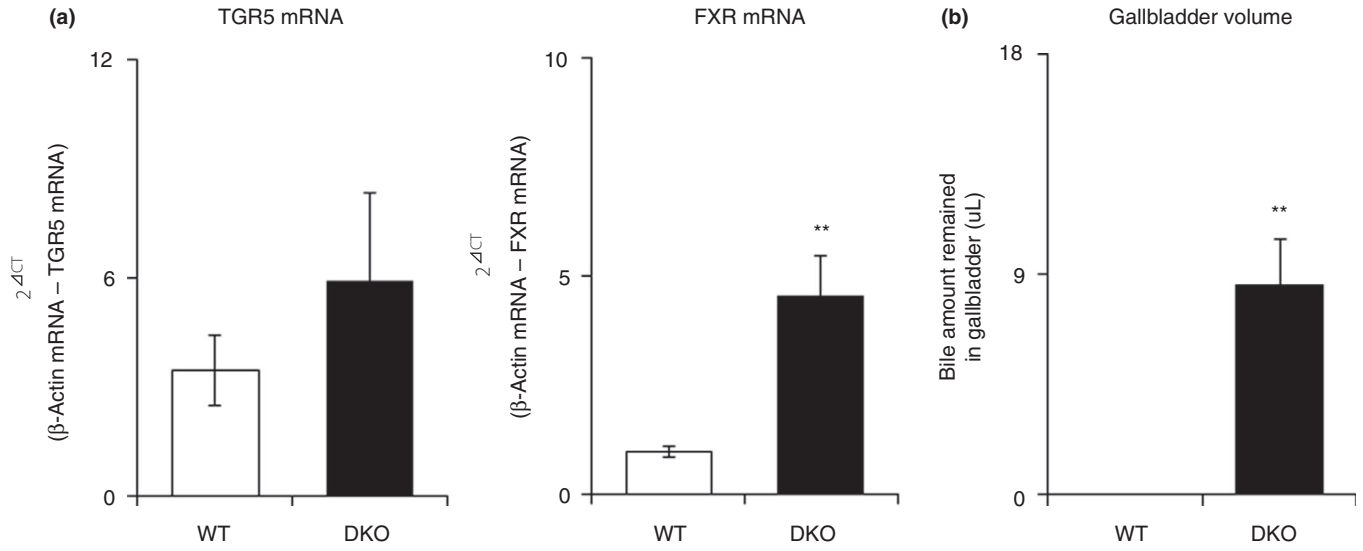

Figure 4 | Expression of bile-associated genes in K cells and gallbladder volume after oil ingestion. The messenger ribonucleic acid (mRNA) expression levels of (a) TGR5 and (b) farnesoid X receptor (FXR) in K cells (n = 5). Gallbladder volume after oil ingestion is expressed by the bile volume remaining in gall bladder (n = 5–13). Wild-type (WT) and double knockout (DKO) mice are represented by white and black bars, respectively. **P < 0.01 versus WT mice.

administration[12]. FABP5 mRNA expression in K cells was not significantly different between the two groups (Figure 3b). GPR119 is a GPCR activated by the long-chain fatty acid derivative, oleoylethanolamide, and is involved in incretin secretion[17,18]. There was no significant difference in GPR119 mRNA levels between the two groups (Figure 3c).

Gene expression of molecules activated by bile acids in the K cells of DKO mice

Bile acids are signaling molecules that activate the transmembrane receptor, TGR5, and the nuclear receptor, farnesoid X receptor (FXR)[19]. TGR5 and FXR are reported to be involved in GLP-1 secretion[20,21]. We previously showed that bile is essential for fat-induced GIP secretion[22]. We therefore evaluated TGR5 and FXR expression in K cells, and bile volume for their involvement in GIP secretion. TGR5 mRNA expression levels in the K cells of DKO mice were comparable to those of WT mice (Figure 4a). Expression levels of FXR mRNA were increased in the K cells of DKO mice (Figure 4b). The bile volume remaining in the gallbladder after oil ingestion was greater in DKO mice than that in WT mice, indicating impairment of gallbladder contraction in DKO mice (Figure 4c).

DISCUSSION

In the present study, we showed that GIP secretion is triggered by oil through GPR120 and GPR40 by *in vivo* data of DKO mice. Ekberg *et al.*[23] reported that olive oil-induced GIP secretion was reduced to 15% in GPR120 and GPR40 DKO mice at 60 min after oil ingestion. The impairment of GIP secretion in DKO mice was even more severe in our present study. Corn oil did not induce GIP secretion in DKO mice at all, and the area under the curve of GIP during OCTTs was reduced to 8% compared with that in WT mice. It is unknown what makes the difference in severity between these two DKO mice lines, but it might be due to their different experimental conditions. In addition, we found that GIP secretion in response to glucose was not impaired in DKO mice, indicating that GPR120 and GPR40 do not affect glucose-stimulated GIP secretion. Thus, GPR120 and GPR40 are essential for oil-induced GIP secretion, but not for glucose-induced GIP secretion. We carried out intraperitoneal oil tolerance tests (Figure S1), and found that intraperitoneal oil administration does not stimulate GIP secretion. This result indicates that GPR120 and GPR40 might be localized at the apical membrane, or that intraperitoneal oil might be not digested into fatty acids.

Bile and pancreatic juice secretion are induced by the intestinal hormone cholecystokinin. Bile is indispensable to oil-induced GIP secretion, as mice subjected to bile duct ligation show no oil-induced GIP secretion at all[22]. Lipase, which is one of the main components in pancreatic juice, is also important, as the lipase inhibitor, orlistat, is reported to reduce incretin secretion in patients with type 2 diabetes[24]. We have reported that GPR40 and GPR120 are involved in cholecystokinin secretion[12]; a defect in contraction of the gallbladder in DKO mice might therefore be involved in the blunted oil-induced GIP secretion. *In vitro* experiments to induce GIP secretion in primary cultured K cells might give us the answer to the question of whether GPR120 and GPR40 are involved in oil-induced GIP secretion directly through intracellular signaling of K cells

or indirectly through defects in bile and pancreatic juice secretion.

We previously reported that GLP-1 levels after corn oil administration were not decreased in GPR120- and GPR40-single knockout mice[12]. Interestingly, corn oil-induced GLP-1 secretion was severely decreased in DKO mice in the present study. Ekberg *et al.* also showed that GLP-1 levels at 60 min after olive oil administration were impaired in GPR120 and GPR40 double-deficient mice, but not in GPR120 and GPR40 single-deficient mice. A compensatory increase of the other GPCR might occur in L cells to ameliorate the impact of the single receptor deficiency. Or there might be some kind of crosstalk between GPR120 and GPR40 involved in L cells. Further studies are required to clarify the effects of the two fatty acid receptors on GLP-1 secretion.

The present study is the first report analyzing K-cell number and gene expressions in K cells under GPR120 and GPR40 DKO condition using GIP reporter (GIP-GFP) mice. K-cell number in the small intestine and GIP mRNA expression in K cells were not different between WT and DKO mice, indicating that GPR120 and GPR40 do not affect GIP production. Furthermore, GPR120 and GPR40 do not alter gene expressions of CD36, FATPs, FABP5 and GPR119, which are involved in fatty acid-induced signal transduction and fatty acids transport.

Bile and its component, bile acids, are important for not only oil digestion and absorption, but also for activation of TGR5 and FXR. In the present study, the expression of FXR mRNA was increased in K cells of DKO mice. FXR is known to decrease the expression of proglucagon mRNA by interfering with carbohydrate-responsive element-binding protein, as well as GLP-1 secretion, by the suppression of the glycolysis pathway, which increases intracellular adenosine triphosphate concentrations[22]. It is unclear whether FXR in K cells regulates GIP expression and intracellular adenosine triphosphate concentrations. As GIP expression in K cells was not altered while FXR expression was upregulated in DKO mice, FXR might have only a small role in GIP expression and secretion. It is reported that FXR mRNA expression in the ileum was increased by cholecystectomy in mice[25]. We have previously shown impaired cholecystokinin secretion and gallbladder contraction in GPR120- and GPR40-single knockout mice, respectively[12]. As DKO mice showed severe impairment in gallbladder contraction, the increase in FXR mRNA expression might reflect the impaired bile secretion.

In conclusion, oil-induced GIP secretion is triggered by the two major fatty acid receptors, GPR120 and GPR40, without a change in K-cell number or K-cell characteristics, such as GIP expression.

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DISCLOSURE

N Inagaki 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 services. The other authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | List of primer sequences for target gene

Figure S1 | Total gastric inhibitory polypeptide (GIP) levels during intraperitoneal corn oil tolerance tests (10 mL/kg bodyweight) in wild-type (WT) mice (n = 6).