Hamaguchi M, et al	Identification of individuals with non-alcoholic fatty liver disease by the diagnostic criteria for the metabolic syndrome	World Journal of Gastroenterology	18	1508-1516	2012
Sato Y, et al	Anks4b, a novel target of HNF4α protein, interacts with GRP78 protein and regulates endoplasmic reticulum stress-induced apoptosis in pancreatic β-cells	J Biol. Chem	287	23236-23245	2012
Ohki T, et al	Identification of hepatocyte growth factor activator (Hgfac) gene as a target of HNF1α in mouse β-cells	Biochem Biophys Res Commun	425	619-624	2012
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Fujita Y, Et al	Successful treatment of reactive hypoglycemia secondary to late dumping syndrome using miglitol	Intern Med	51	2581-2585	2012

Fujishim Y, et al	Efficacy of liraglutide, a glucagon-like peptide-1 (GLP-1) analogue, on body weight, eating behavior, and glycemic control, in Japanese obese type 2 diabetes	Cardiovasc Diabetol	11	107	2012

著書

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塩谷真由美、他	遺伝子異常による糖尿病／耐糖能異常 スマートな糖尿病診断と治療の進め方	中山書店	69*76	2011
堀川幸男	Annual Review 糖尿病・代謝内・分泌 2011 糖尿病ゲノム研究の現状と展望	中外医学社	67-74	2011

【３】研究成果の刊行物・別刷

Synergistic effect of α-glucosidase inhibitors and dipeptidyl peptidase 4 inhibitor treatment

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ABSTRACT

Monotherapy of α-glucosidase inhibitor (α-GI) or dipeptidyl peptidase 4 (DPP4) inhibitor does not sufficiently minimize glucose fluctuations in the diabetic state. In the present study, we evaluated the combined effects of various of α-GI inhibitors (acarbose, voglibose or miglitol) and sitagliptin, a DPP4 inhibitor, on blood glucose fluctuation, insulin and active glucagon-like peptide-1 (GLP-1) levels after nutriment loading in mice. Miglitol and sitagliptin elicited a 47% reduction ($P < 0.05$) of the area under the curve of blood glucose levels for up to 2 h after maltose-loading, a 60% reduction ($P < 0.05$) in the range of blood glucose fluctuation, and a 32% decrease in plasma insulin compared with the control group. All three of the combinations elicited a 2.5–4.9-fold synergistic increase in active GLP-1 ($P < 0.05$ *vs* control). Thus, combined treatment with the α-GI miglitol, which more strongly inhibits the early phase of postprandial hyperglycemia, and DPP4 inhibitor yields both complementary and synergistic effects, and might represent a superior anti-hyperglycemic therapy. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00081.x, 2010)

KEY WORDS: Postprandial hyperglycemia, Insulin, Glucagon-like peptide-1

INTRODUCTION

Although active intervention for postprandial hyperglycemia by acarbose, an α-glucosidase inhibitor (α-GI), prevents development of cardiovascular events[1], it is also important to flatten the postprandial glucose fluctuation to prevent macroangiopathy. α-GI not only inhibits the rapid elevation of postprandial blood glucose level without excessive insulin secretion, but also enhances active glucagon-like peptide-1 (GLP-1) secretion[2]. In contrast, the dipeptidyl peptidase 4 (DPP4) inhibitor, sitagliptin, increases insulin secretion and reduces late-phase elevation of postprandial blood glucose level. We hypothesized that a combination of α-GI and sitagliptin might yield a greater minimizing effect on blood glucose fluctuation while conserving insulin secretion and enhancing active GLP-1 secretion to prevent atherosclerosis[3–5]. Recently, the combined effects of voglibose, an α-GI, and a DPP4 inhibitor on plasma insulin and active GLP-1 levels were reported in mice[6,7]. However, comparison of the combined effect of various α-GIs and sitagliptin on blood glucose fluctuation has not been reported.

In the present study, we showed that combination therapy of α-GIs and sitagliptin can yield complementary and synergistic beneficial effects in mice.

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MATERIALS AND METHODS

Because α-GIs are inhibitors of α-glucosidase, which converts disaccharide to glucose, 7–9-week-old male C57BL/6J mice (Charles River Japan, Tokyo, Japan) were subjected to an overnight fast and orally loaded with 2.5 g/kg of maltose. Blood was collected from the end of the tail just before loading until 2 h after loading, and blood glucose level was measured using the glucose dehydrogenase method. The area under the curve of blood glucose levels for up to 2 h after maltose-loading ($\Delta AUC_{0-2\ h}$) was calculated using the trapezoid method. The range of blood glucose fluctuation was determined as the difference between the maximal and minimal blood glucose levels for up to 2 h.

To evaluate initial insulin-secreting capacity, plasma insulin concentration in blood collected from the end of the tail at 0.25 h after loading was measured using an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Ten-week-old male C57BL/6J mice freely fed a high-fat diet (D12492 Rodent Diet; Research Diets, New Brunswick, NJ, USA) for 6 weeks were orally loaded with 10 mL/kg of enteral nutrition (Ensure H; Abbot Japan, Tokyo, Japan) to stimulate GLP-1 secretion. To analyze the delayed effect of GLP-1 secretion by α-GI, blood was collected after 0.5 h from the abdominal vein using a syringe containing diprotin A and EDTA at a final concentration of 3 mmol/L and 0.15%, respectively. The concentration of plasma active GLP-1 was measured using an ELISA kit (Millipore Corporation, Billerica, MA, USA).

α-Glucosidase inhibitors (acarbose 10 mg/kg, voglibose 0.1 mg/kg or miglitol 3 mg/kg) was given orally at the time of maltose or enteral nutrition-loading, and sitagliptin (0.3 mg/kg)

was given 0.5 h before oral loading. Test doses of α-GIs were determined from the ED_{50} doses of the inhibition effect on sucrose or maltose-loading in normal rats, respectively.

The experiment was carried out according to the guidelines of Gifu University. Results are expressed as mean ± SD. Significance of difference among groups was analyzed using Dunnett's or Tukey's multiple comparison test based on ANOVA. *P*-values of <0.05 were considered to show statistical significance.

RESULTS

In the maltose-loading test, treatment with each of the three α-GIs alone significantly suppressed the blood glucose peak level at 0.5 h. Administration of miglitol alone significantly suppressed the blood glucose elevation as early as 0.25 and 0.5 h, and delayed the blood glucose peak until 1 h after loading. With administration of sitagliptin alone, the blood glucose level peaked at 0.25 h and was significantly lower at 0.5 and 1 h. Consequently, a complementary suppression of blood glucose elevation was observed at 0.25, 0.5 and 1 h in the combined miglitol and sitagliptin group (Figure 1c). As a result, the $\Delta AUC_{0-2\,h}$ of the blood glucose concentration of the group receiving miglitol alone was almost equal to that with acarbose or voglibose alone. The rate of decrease of the $\Delta AUC_{0-2\,h}$ of the blood glucose concentration in the miglitol and sitagliptin combination group was 47% ($P < 0.05$) compared with the control group, which was larger than that observed in the combined groups with acarbose or voglibose at the tested doses (Table 1). Similarly, combined treatment of miglitol and sitagliptin showed a 60% reduction ($P < 0.05$) of the range of blood glucose fluctuation compared with the control group, and also a significant decrease compared with miglitol or sitagliptin alone (Table 1).

Enhancement of plasma insulin occurred with sitagliptin at 0.25 h after loading (1.6–2.0-fold). In contrast, miglitol alone or combined treatment with miglitol and sitagliptin decreased the plasma insulin concentration by 39 or 32% compared with the control group, respectively (Table 1).

When enteral nutrition was orally loaded in mice fed a high-fat diet, the plasma active GLP-1 concentration was increased by 1.5–1.9-fold after administration of sitagliptin compared with the control group. In contrast, a synergistic increase in the GLP-1 concentration was observed after treatment with a combination of α-GI and sitagliptin (2.5–4.9-fold, $P < 0.05$ *vs* control; Table 1).

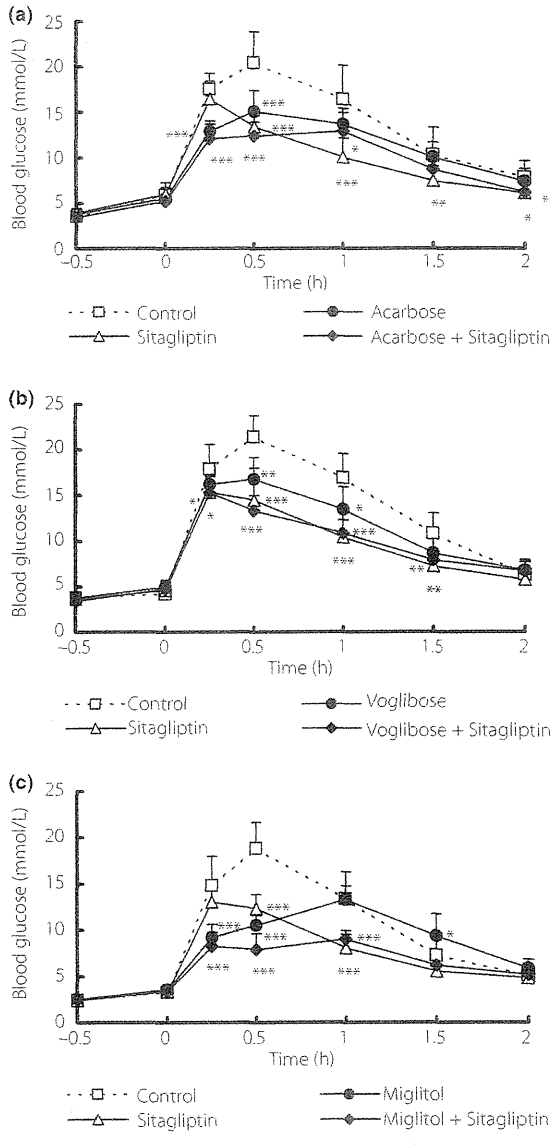

Figure 1 | Blood glucose profiles after oral administration of α-glucosidase inhibitor (α-GI) and sitagliptin (0.3 mg/kg) in maltose-loaded normal mice. (a) Acarbose, 10 mg/kg; (b) voglibose, 0.1 mg/kg; (c) miglitol, 3 mg/kg. Each value represents mean ± SD of 8–10 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control group using to Dunnett's multiple comparison test.

DISCUSSION

Recently, suppression of postprandial hyperglycemia has come to be considered important for prevention of atherosclerosis[3]. Repeated episodes of blood glucose fluctuation accelerate the adhesion of monocytes to vascular endothelial cells and enhance the development/progression of atherosclerosis[8]. In the present study, the combined administration of α-GI and sitagliptin complementarily lowered the blood glucose level. Furthermore, the combination of miglitol with sitagliptin significantly minimized blood glucose fluctuations. Thus, such combined treatment should reduce the risk of atherosclerosis.

The suppression of postprandial hyperglycemia by α-GI alone and combination treatment with sitagliptin enables insulin secretion to be conserved, and therefore can be expected to mitigate dysfunction of pancreatic β-cells[9]. In contrast, sitagliptin decreases the blood glucose level by enhancing insulin secretion. Thus, long-term administration of this agent might create a

Table 1 | Incremental blood glucose, range of glucose fluctuation and plasma insulin in maltose-loaded mice and plasma active glucagon-like peptide-1 in enteral nutrition-loaded mice

Test compound	Blood glucose concentration $\Delta AUC_{0-2\ h}$		Range of glucose fluctuation	Plasma insulin at 15 min after maltose loading	Plasma active GLP-1 at 30 min after EN loading
	(h*mmol/L)	Inhibition rate (%)	mmol/L	pmol/L	pmol/L
Control	20.9 ± 4.6^{a}	–	15.3 ± 2.6^{a}	111.9 ± 44.8^{ab}	2.18 ± 0.90^{a}
Acarbose, 10 mg/kg	15.6 ± 2.6^{b}	25.4	10.0 ± 2.5^{b}	89.5 ± 25.8^{a}	2.37 ± 0.76^{a}
Sitagliptin, 0.3 mg/kg	12.3 ± 3.3^{b}	41.1	10.9 ± 1.7^{b}	173.9 ± 53.4^{b}	3.33 ± 1.44^{a}
Combination	13.8 ± 2.3^{b}	34.0	8.5 ± 2.3^{b}	118.8 ± 68.9^{ab}	10.76 ± 6.34^{b}
Control	20.9 ± 3.2^{a}	–	17.2 ± 2.4^{a}	113.7 ± 48.2^{a}	2.23 ± 1.86^{a}
Voglibose, 0.1 mg/kg	16.1 ± 3.2^{b}	23.0	12.3 ± 1.7^{b}	111.9 ± 17.2^{a}	2.78 ± 1.23^{a}
Sitagliptin, 0.3 mg/kg	12.7 ± 2.9^{b}	39.2	11.6 ± 2.4^{b}	229.0 ± 93.0^{b}	3.54 ± 2.33^{ac}
Combination	13.6 ± 1.3^{b}	34.9	10.7 ± 1.8^{b}	142.9 ± 43.1^{a}	5.52 ± 2.34^{bc}
Control	17.9 ± 3.6^{a}	–	15.5 ± 2.6^{a}	158.9 ± 44.8^{a}	2.37 ± 1.33^{a}
Miglitol, 3 mg/kg	14.4 ± 1.7^{b}	19.6	9.7 ± 1.3^{b}	96.4 ± 18.9^{a}	3.29 ± 1.13^{a}
Sitagliptin, 0.3 mg/kg	11.5 ± 1.6^{bc}	35.8	10.0 ± 1.8^{b}	252.5 ± 86.1^{b}	4.62 ± 3.06^{a}
Combination	9.4 ± 1.3^{c}	47.5	6.2 ± 1.3^{c}	107.3 ± 20.7^{a}	11.22 ± 9.72^{b}

Maltose-loading tests used for normal mice. Enteral nutirition (EN)-loading tests used for mice fed high-fat diet for 6 weeks. Each value represents the mean ± SD of 8–10 mice. Means with different letters (a, b, and c) are significantly different at $P < 0.05$ by Tukey's multiple comparison test. $AUC_{0-2\ h}$, the area under the curve of blood glucose levels for up to 2 h after maltose-loading; GLP-1, glucagon-like peptide-1.

burden on pancreatic β-cells. In addition, a correlation between hyperinsulinemia and the level of high-sensitivity C-reactive protein (CRP), a marker of inflammation, has been reported[10]. Because the CRP elevation is related to coronary heart disease, stroke and mortality[5], insulin secretion should be reduced as much as possible. In the present study, when α-GI and sitagliptin were used in combination, insulin secretion was suppressed to a level almost the same as that with α-GI alone. Furthermore, after chronic combined treatment of miglitol and sitagliptin for 8 weeks in high-fat diet fed mice, the elevation of fasting plasma insulin level was significantly suppressed compared with that of control mice (normal diet group: 137.8 ± 84.4 pmol/L; high-fat control group: 253.1 ± 72.3 pmol/L, $P < 0.05$ *vs* normal diet group; miglitol alone group: 189.4 ± 110.2 pmol/L; sitagliptin alone group: 359.9 ± 187.7 pmol/L; combination group: 161.9 ± 84.4 pmol/L, $P < 0.05$ *vs* high fat control; Y.H. and J.T., unpublished data). Thus, combined treatment might reduce risk of the development/progression of atherosclerosis as well as dysfunction of pancreatic β-cells.

In patients with type 2 diabetes, miglitol has been reported to increase plasma active GLP-1 levels[2,11]. GLP-1 has several physiological activities, including a trophic effect on the pancreatic islets and suppression of gastric emptying and appetite[12], in addition to enhancement of insulin secretion and inhibition of glucagon secretion. When combined with sitagliptin, each α-GI tested increased the active GLP-1 concentration synergistically. The GLP-1 secretion induced by α-GI might result from delayed absorption of carbohydrate into the lower parts of the digestive tract[13], although the details of this mechanism remain unclear.

In conclusion, combined treatment with α-GI miglitol, which more strongly inhibits the early phase of postprandial hyperglycemia, and sitagliptin can yield complementary and synergistic effects and therefore might represent a better antihyperglycemic therapy.

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Krüppel-like factor-10 is directly regulated by carbohydrate response element-binding protein in rat primary hepatocytes

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ABSTRACT

Krüppel-like factor (KLF)-10, is a circadian transcriptional regulator, which links the molecular clock to energy metabolism in the liver. Recently, it was reported that *Klf-10* expression is induced by glucose stimulation in mouse hepatocytes. We previously reported that carbohydrate response element-binding protein (ChREBP) plays an important role in the regulation of hepatic lipogenic gene expression. Here, we investigate whether ChREBP, a glucose-activated transcription factor, directly regulates *Klf-10* mRNA expression in rat primary hepatocytes. We found that both glucose stimulation and adenoviral overexpression of ChREBP induce *Klf-10* mRNA expression in rat primary hepatocytes in a dose-dependent manner. Conversely, overexpression of dominant-negative Max-like protein inhibits glucose-induction expression of *Klf-10* mRNA. Deletion analysis using rat *Klf-10* promoter in the pGL3 vector combined with a chromatin immunoprecipitation assay against the anti-ChREBP antibody demonstrated that the carbohydrate response element is located between −125 bp and −109 bp in the rat *Klf-10* promoter. Conversely, adenoviral overexpression of KLF-10 partly inhibits glucose induction of ChREBP target genes in primary hepatocytes. In conclusion, these data suggest that crosstalk between ChREBP and KLF-10 is involved in the regulation of the lipogenic pathway.

1. Introduction

In the liver, *de novo* lipogenesis plays an important role in the development of metabolic syndrome and fatty liver disease [1]. Hepatic gene expression in the glycolytic and lipogenic pathways is regulated by insulin and glucose at the transcriptional level. Insulin activates sterol regulatory element-binding protein-1c, whereas glucose activates carbohydrate response element-binding protein (ChREBP) [1]. ChREBP is a basic helix–loop–helix leucine zipper transcription factor of the Myc/Max/Mad superfamily [2] and is mainly expressed in the liver, kidney, muscle, and white and brown adipose tissues [3,4]. ChREBP and Max-like protein (MLX) form a heterodimeric complex and induce ChREBP target gene expression through the binding of ChREBP to carbohydrate response element (ChoRE) motifs in the promoters of lipogenic genes [2]. Glucose activates ChREBP transactivity, inducing hepatic glycolytic and lipogenic gene expression through dephosphorylation of ChREBP by protein phosphatase 2A activation [2]. In contrast, cAMP and AMP inhibit ChREBP transactivity through the phosphorylation of ChREBP by protein kinase A and AMP-activated kinase activation [2]. ChREBP mediates conversion of excess glucose to triglycerides in the liver [5]. The inhibition of ChREBP prevents glucose intolerance, fatty liver, and obesity in *ob/ob* mice, and thus, is a potential therapeutic target for the treatment of obesity-related metabolic disorders [6,7].

Krüppel-like factors (KLFs) are zinc finger-containing transcription factors that regulate proliferation, differentiation, development, and programmed cell death [8]. KLF-10 was initially identified as a primary transforming growth factor-β-inducible early gene in human osteoblasts [9]. Defects in *Klf-11* are the cause of maturity-onset diabetes of the young type 7 [10,11]. KLF-10 and KLF-11 share an amino-terminal transcription repression domain, which is also required for the growth-suppressive effect of KLF-11 [8]. Recently, it was reported that *Klf-10* mRNA expression is regulated by CLOCK/BMAL1 heterodimers and that KLF-10 itself suppresses lipogenic gene expression [12]. In *Klf-10* knockout mice, it was reported that male *Klf-10*$^{-/-}$ mice displayed postprandial and fasting hyperglycemia, whereas *Klf-10*$^{-/-}$ females were normoglycemic but displayed higher plasma triglyceride concentrations [12]. In addition, we and other groups have demonstrated that glucose stimulation induces *Klf-10* mRNA expression, both in mouse primary hepatocytes and Rat-1 fibroblasts [12–14].

We recently reported that ChREBP directly regulates the gene expression of *Bhlhb2* (also known as the clock gene *DEC1*), which is regulated by both glucose stimulation and by heterodimers of the molecular clock proteins CLOCK/BMAL1 in the liver [15]. In addition, we demonstrated that ChREBP and BHLHB2 coordinately regulate *de*

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novo lipogenesis in rat primary hepatocytes [16]. Similarly, KLF-10 expression is also regulated by CLOCK/BMAL1 proteins and glucose stimulation. Therefore, we investigated whether glucose regulates KLF-10 expression through the activation of ChREBP using the same mechanism as BHLHB2.

In this study, we aimed to determine whether ChREBP regulates *Klf-10* expression by directly binding to ChoRE in the rat *Klf-10* promoter region. Elucidation of the relationship between ChREBP and KLF-10 in the regulation of lipogenesis will significantly enhance our understanding of the regulation of lipogenic gene expression, and in turn, the pathology of metabolic syndrome.

2. Materials and methods

2.1. Materials, tissue culture, hepatocyte isolation

Protocols for all animal experiments were approved by the Institutional Animal Care and Use Committee of Gifu University Medical School (code numbers: 22–26). Rat primary hepatocytes were isolated and cultured from 6-week-old male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) as previously described [11,12].

2.2. Construction of plasmid and adenovirus vectors

We used pcDNA-daChREBP, Ad-daChREBP, Ad-dnMLX, and pGL4 TK RLuc vectors, as previously described [17,18]. For the deletion analysis, a series of pGL3-KLF-10 vectors were constructed as follows: fragments of the native 5′ sequence flanking the rat *Klf-10*, representing −808, −646, −346, −218, −108, and −48 bp relative to the transcription start site of the rat *Klf-10* (positions: −808/+56, −646/+56, −346/+56, −218/+56, −108/+56, and −48/+56), were cloned upstream of the luciferase gene into the basic pGL3 luciferase reporter vector (Promega; Wisconsin, USA). Two fragments, 3xK10-1 E1 (−133/−117 bp) and 3xK10-2 (−125/−109 bp), were cloned into pGL3 vectors upstream of the TK promoter.

Rat KLF-10 cDNA-expressing adenovirus (Ad-KLF-10) was constructed as follows: rat *Klf-10* full-length cDNA was cloned using PrimeSTAR® HS DNA polymerase reagents (Takara Bio Inc., Shiga, Japan). The PCR fragment was then ligated into the pENTR vector (Invitrogen; California, USA). Recombination of the adenovirus and pENTR-KLF-10 vector was performed according to the manufacture's protocol to produce Ad-KLF-10. All plasmid and adenovirus vectors were verified by sequencing analysis.

2.3. Treatment with recombinant adenovirus

Rat primary hepatocytes were cultured in 6-well plates in 2 ml Dulbecco's modified Eagle's medium (DMEM). After infection with adenovirus bearing green fluorescent protein (GFP), either dominant-active ChREBP (daChREBP), dominant-negative MLX (dnMLX), or KLF-10 was used to infect hepatocytes for 2 h. The medium was then removed and the hepatocytes were incubated in a fresh medium with either 2.5 or 25 mM glucose for 18 h. Cells were then collected and used for RNA extraction, cDNA synthesis and RT-PCR analysis as described previously [17,18].

2.4. Mammalian transfection and reporter assay

Rat primary hepatocytes were cultured in 6-well plates in 2 ml DMEM without antibiotics. The cells were transfected with 4 μl of Lipofectamine® 2000 transfection reagent, 1.4 μg of the series of pGL3-KLF-10, and 0.1 μg of pGL4 TK RLuc vector [12]. After 24 h of incubation with either 2.5 mM or 25 mM glucose, the cells were collected and luciferase activity was measured using the Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA) according to the manufacture's protocol. To determine the glucose dependency of the glucose response region in rat *Klf-10* promoter, cells were transfected with 1.0 μg of the pGL3 promoter 3xK10-1 or 3xK10-2 putative ChoRE vectors, 0.1 μg of pGL4 TK RLuc vectors, and 0.4 μg of pcDNA6.2 empty vector or pcDNA-daChREBP vector to adjust total DNA. After 24 h of incubation with various glucose concentrations, the cells were collected for measurement of luciferase activity.

2.5. ChIP assay

A ChIP assay was performed with anti-ChREBP antibody (Novus Biologicals, Littleton, CO) on formaldehyde cross-linked hepatocytes using Magna ChIP G (Millipore, Temecula, CA). Rat hepatocytes were isolated and cultured in 10-cm culture dishes in DMEM supplemented with 10% FCS, 100 nM insulin, 100 nM dex, 10 nM T_3 and 100 μg/ml pen/strep for 4 h and then for 2 h in DMEM supplemented with 10 nM T_3 and 100 μg/ml pen/strep. After the incubations, 10% formaldehyde (270 μl) was added to the culture dishes and the cells were used in the chromatin immunoprecipitation (ChIP) assay according to the manufacturer's protocol. The purified DNA was dissolved in Tris/EDTA (50 μl, pH 8.0) and used with gene specific primers for the PCR. Buffer only and rabbit normal IgG (Wako Chemical) were used as the negative control. Immunological chromatin samples were amplified by PCR using the following primers: 5′-FAM-CTCCACTGACAGAGCTTCTTG-CAGCCG-TAMRA-3′ (probe), 5′-ACGGTGCCTCGGGTTGTG-3′ (Forward), and 5′-TCGACCAATCAGCGGTAAAGG-3′ (Reverse).

2.6. Data presentation and statistical methods

All data are expressed as mean ± SD. The listed *n* values represent the number of single experiments performed (each experiment was duplicated). Comparison between the two groups was performed using the student *t*-test, and comparison between multiple groups was performed using the Tukey–Kramer test. A P value of <0.05 was regarded statistically significant.

3. Results

3.1. KLF-10 is a ChREBP target gene in rat primary hepatocytes

First, we examined the tissue distribution of *Klf-10* mRNA expression in C57BL/6J mice. In 6-week-old male C57BL/6J mice *Klf-10* mRNA is highly expressed in the liver and muscle (Supplementary Fig. 1A). Next, we tested whether *Klf-10* mRNA expression is higher in metabolic syndrome model animals. *Klf-10* mRNA expression is higher in the liver of *ob/ob* mice compared with C57BL/6J mice (Supplementary Fig. 1B). These data suggested that glucose induces rat *Klf-10* mRNA expression in the liver. Furthermore, we confirmed that *Klf-10* mRNA, but not *Chrebp* mRNA shows a circadian expression profile in the liver. This suggests that *Klf-10* mRNA expression is controlled by CLOCK/BMAL1 and is a circadian transcriptional modulator (Supplementary Fig. 1C).

Next, we tested whether glucose stimulation and adenoviral overexpression of ChREBP induced *Klf-10* mRNA expression. In rat primary hepatocytes, glucose activated *Klf-10* mRNA expression in a dose-dependent manner. There was an 8-fold increase in *Klf-10* mRNA with 25 mM glucose compared with 2.5 mM glucose (Fig. 1A). Similarly, the level of *Klf-10* mRNA expression was upregulated by overexpression of daChREBP (Fig. 1B). Increasing the dose (5, 10, 20, and 50 MOI) of adenovirus bearing daChREBP cDNA resulted in a corresponding increase in *Chrebp* mRNA: 2.5-, 9.4-, 38-, and 131-fold compared with endogenous *Chrebp* mRNA

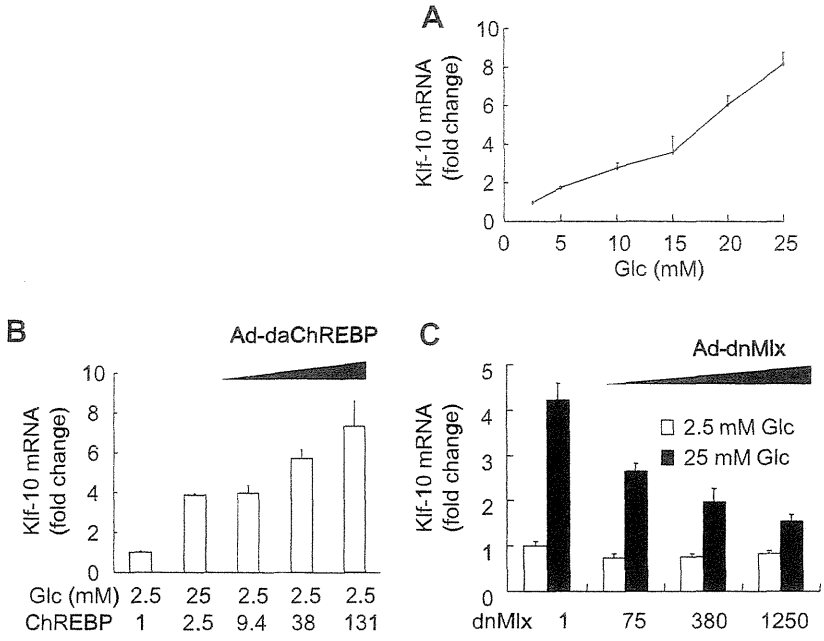

Fig. 1. Regulation of the rat *Klf-10* promoter by glucose (A) Dose-dependent effects of glucose on *Klf-10* mRNA expression in rat primary hepatocytes. Primary hepatocytes isolated from 6-week-old male Wister rats were cultured for 18 h in culture medium containing 2.5, 5.0, 10, 15, 20 or 25 mM glucose. Relative mRNA levels were determined by real-time RT-PCR and normalized to rat *RNA polymerase II* (*Pol2*) mRNA as the invariant control. The change in expression level of each normalized enzyme mRNA level was determined with reference to the value for hepatocytes at 2.5 mM glucose, which was arbitrarily defined as 1. (B) Adenoviral overexpression of daChREBP activates rat *Klf-10* mRNA expression. In hepatocytes infected with 5, 10, 20, and 50 MOI of Ad-daChREBP or 50 MOI of Ad-GFP, *Chrebp* mRNA was increased 2.5-, 9.4-, 38-, and 131-fold, respectively. *Klf-10* mRNA levels were analyzed by TaqMan® RT-PCR and corrected with rat *Pol2*. Data are mean ± SD ($n = 3$) of two independent experiments. (C) Adenoviral overexpression of dnMLX activates rat *Klf-10* mRNA expression. In hepatocytes infected with 5, 20, and 50 MOI of Ad dnMLX or 50 MOI of Ad-GFP, *dnmlx* mRNA was increased 75-, 380-, and 1250-fold, respectively. *Klf-10* mRNA levels were analyzed by TaqMan® RT-PCR and corrected with rat *Pol2*. Data are mean ± SD ($n = 3$) of two independent experiments.

(Fig. 1B). Consistent with these data, adenoviral overexpression of dnMLX inhibited glucose-induced *Klf-10* mRNA expression in rat primary hepatocytes (Fig. 1C). These data provide indirect evidence that glucose induces *Klf-10* mRNA expression through the glucose-activated transcription factor ChREBP.

3.2. The identification of ChoRE in rat KLF-10 promoter

ChREBP is activated by glucose and binds to ChoRE of the lipogenic gene promoter region. To test whether ChREBP directly activates the rat *Klf-10* promoter, we ran a reporter assay using a series of pGL3-*KLF-10* deletion constructs (−808, −646, −346, −218, −108, and −48 bp). Glucose stimulation increased the transactivity of the −808, −646, −346, and −218 bp constructs, all of which contain putative ChoREs (Fig. 2A). These data suggested that the ChoRE in the rat *Klf-10* promoter region is located between −218 and −108 bp. By referring to the consensus sequence of ChoREs (CAYGNGN5CNCRTG), we found two putative ChoREs (K10-1: −133/−117 bp and K10-2: −125/−109 bp) in the rat *KLF-10* promoter region (Supplementary data Fig. 2A).

We used pGL3 promoter vectors, containing in tandem the fragments 3xK10-1 (−133/−117 bp) and 3xK10-2 (−125/−109 bp), to test whether these putative ChoREs had the ability to respond to glucose. The luciferase activity of PGL3 3xK10-1 at a glucose concentration of 25 mM was similar to that of the control cells; however, the luciferase activity of PGL3 3xK10-2 at a glucose concentration of 25 mM was >80-fold higher than that of control cells (Fig. 2B). In agreement with this, da-ChREBP overexpression caused >30-fold increase in the luciferase activity of PGL3 3xK10-2 in rat primary hepatocytes (Fig. 2C). Similarly, glucose and daChREBP overexpression dose-dependently induced luciferase activity of PGL3 3xK10-2 (Supplementary Fig. 2B and C). Thus, one of the two putative ChoREs (K10-2) in rat *Klf-10* promoter possessed glucose responsiveness.

Consistent with these data, a chromatin immunoprecipitation (ChIP) assay against anti-ChREBP antibody showed ChREBP directly bound to the ChoRE of the rat *KLF-10* promoter (Fig. 2D). L-type pyruvatekinase (*Lpk*), a ChREBP target gene found in the rat liver, was used as a positive control.

3.3. Effects of KLF-10 overexpression on ChREBP and its target genes

Recently, it was shown that deletion of *KLF-10* augmented the glucose induction of *Lpk*, fatty acid synthase (*Fasn*) and Acetyl-CoA Carboxylase 1 (*Acc1*) in rat primary hepatocytes. Adenoviral overexpression of KLF-10 caused a 500-fold increase in *Klf-10* mRNA expression and inhibited glucose-stimulated ChREBP target genes, such as *Lpk*, *Fasn*, and *Acc1*, in rat primary hepatocytes (Fig. 3). *Smad-7* is a well-known KLF-10 target gene and was used as a positive control. In line with previous data, glucose stimulation and overexpression of KLF-10 inhibit *Smad7* expression in rat hepatocytes. Interestingly, adenoviral overexpression of KLF-10 causes a decrease in *Chrebp* mRNA expression in rat primary hepatocytes (Fig. 3). This suggests that KLF-10 partly inhibits the glucose induction of ChREBP target gene expression in rat primary hepatocytes.

4. Discussion

In this study, we demonstrated that the glucose-activated transcription factor ChREBP regulates rat *Klf-10* expression by directly binding to ChoRE in rat *Klf-10* promoter region. Conversely, KLF-10 partly suppresses glucose induction of ChREBP target gene expression in the liver. These data suggest that crosstalk between ChREBP and KLF-10 is involved in *de novo* lipogenesis.

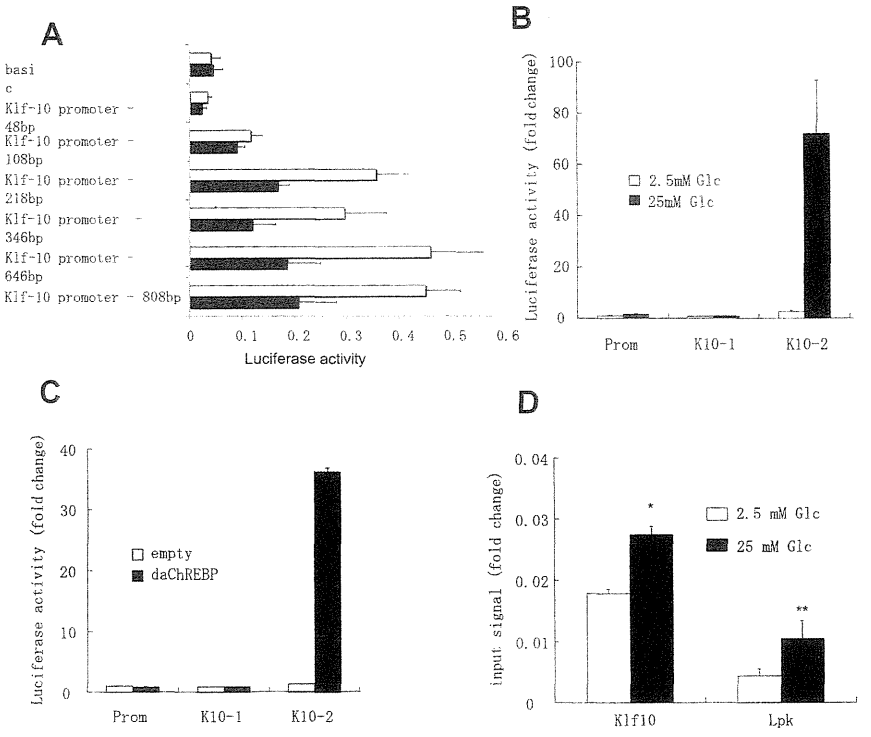

Fig. 2. Direct regulation of the rat *Klf-10* promoter by ChREBP (A) A schematic representation of the rat *Klf-10* promoter luciferase deletion constructs and the effect of thes deletions on relative luciferase activity at low (2.5 mM) or high (25 mM) glucose concentrations. A range of reporter gene plasmids (−808, −646, −346, −218, −108, an −48 bp) were cotransfected into rat hepatocytes with pGL4.74 [hRLuc/TK] as a reference and luciferase activity was normalized to *Renilla* luciferase activity. The values ar represented as mean ± SD ($n = 6$). The figure represents data from two independent experiments. (B) Putative ChoRE in the rat *Klf-10* is a response element activated b glucose and ChREBP. The pGL3 promoter vector containing tandem 3xK10-1 or 3xK10-2 putative ChoREs was cotransfected into rat primary hepatocytes with pGL4.74[hRLuc TK]. Total DNA was adjusted with pcDNA 6.2 empty vectors. The relative luciferase activity was expressed as an *n*-fold change with reference to the pcDNA6.2 empty vecto Data are mean ± SD ($n = 6$) of two independent experiments. (C) Putative ChoRE in the rat *Klf-10* is a response element activated by glucose and ChREBP. The pGL3 promote vector containing tandem 3xK10-1 or 3xK10-2 putative ChoREs was cotransfected into rat primary hepatocytes with pGL4.74[hRLuc/TK]. Total DNA was adjusted with pcDN 6.2 empty vectors. The relative luciferase activity was expressed as an *n*-fold change with reference to the pcDNA6.2 empty vector. Data are mean ± SD ($n = 6$) of tw independent experiments. Cells were incubated for 4 h in medium and subjected to a ChIP assay using the anti-ChREBP or anti-MLX antibodies. Buffer alone and nonspecifi rabbit IgG were used as negative controls. Immunoprecipitated samples were subjected to PCR using primers to amplify ChoRE-containing regions of the rat *Klf-10* or *Lp* promoters. All experiments were performed in duplicate.

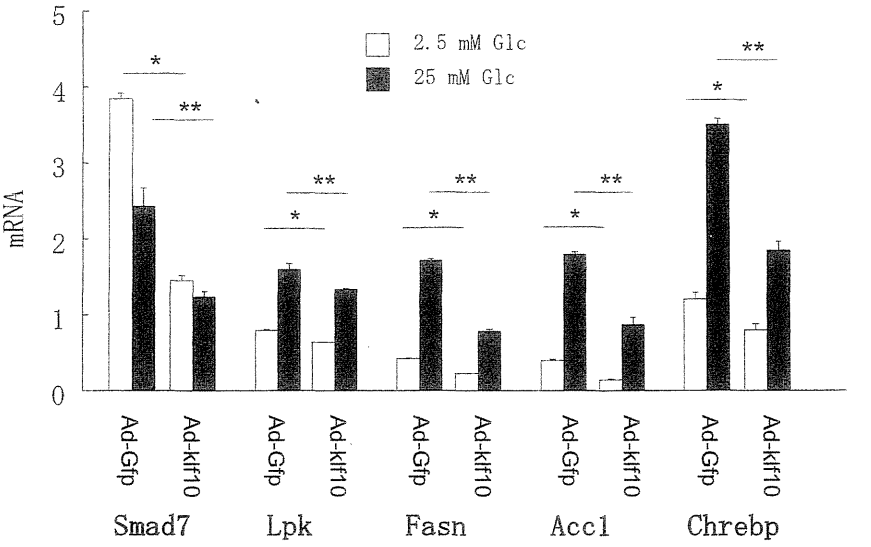

Fig. 3. Adenoviral overexpression of KLF-10 causes decreased ChREBP target gene expression. KLF-10 dose-dependently inhibited the glucose induction of ChREBP targ gene expression in rat primary hepatocytes. In hepatocytes infected with 50 m.o.i. of Ad-KLF-10 adenovirus, *Klf10* mRNA expression increased 500-fold. *Smad7*, *Klf-10*, *Fas Acc1*, *Lpk*, and *Chrebp* mRNA levels were detected by TaqMan® RT-PCR and corrected with *Pol2* mRNA. Data are represented as mean ± SD.

First, *Klf-10* mRNA is expressed in the liver and muscle and is more highly expressed in the liver of genetically obese mice compared with control mice. Similarly, our group and others hav reported that *Chrebp* mRNA is expressed in the lipogenic tissue

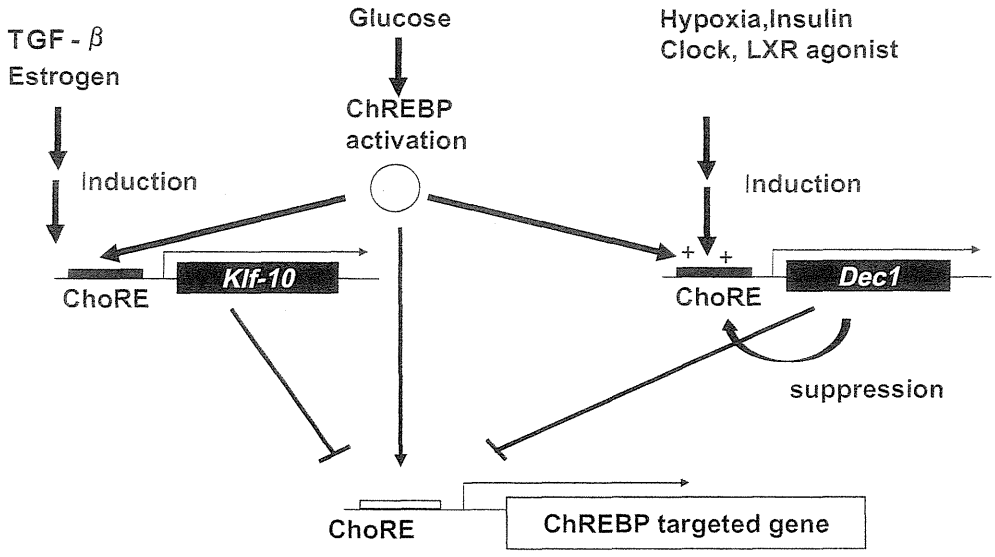

Fig. 4. A schematic representation of the feedback loop model for the *de novo* coordinate regulation of lipogenesis. In rat hepatocytes, ChREBP induces the transcription repressors such as KLF-10 and BHLHB2. In turn, these repressors inhibit the glucose induction of ChREBP target gene expression (*L-pk*, *Fasn*, and *Acc1*). ChREBP and these transcriptional repressors constitute a negative feedback loop in the regulation of lipogenic gene expression.

such as the liver, kidney, and muscle and is more highly expressed in the liver of genetically obese mice. These data suggest that KLF-10 and ChREBP coordinately regulate lipogenic gene expression in the liver. Furthermore, *Klf-10* shows a circadian expression profile in the liver. In accordance with this data, circadian expression of *Klf-10* mRNA is evident in C57BL/6J mice; however, circadian expression of *Chrebp* mRNA was not observed. These results suggest that the regulation of *Klf-10* mRNA expression by ChREBP is independent of the CLOCK/BMAL1 system.

Second, we demonstrated that ChREBP directly induces *Klf-10* mRNA expression by binding to ChoRE in rat *KLF-10* promoter region. We demonstrated that glucose stimulation and overexpression of daChREBP can induce *Klf-10* mRNA expression and that overexpression of dnMLX inhibits the glucose induction of *Klf-10* by inhibiting the formation of a ChREBP/MLX complex. Consistent with these results, other groups have reported that glucose induces *Klf-10* mRNA expression in mice primary hepatocytes Therefore, glucose induces *Klf-10* mRNA expression through ChREBP transactivation. Moreover, using a series of PGL3-KLF-10 promoter deletion constructs, we identified that the ChoRE is located between −125 and −109 bp in the *KLF-10* promoter region. In agreement with this data, the reporter assay and the ChIP assay revealed that this ChoRE is responsive to glucose and that ChREBP binds directly to the ChoRE (−125 bp to −109 bp) *in vivo*. Moreover, 49-fold overexpression of daChREBP caused a 5-fold increase in *Klf-10* mRNA expression in the liver of mice intravenously infected with Ad-daChREBP compared with those infected with Ad-GFP (data not shown). Thus, ChREBP induces *KLF-10* expression *in vitro* and *in vivo*. Collectively, these data suggest that glucose directly regulates rat *KLF-10* expression through ChREBP transactivation in rat primary hepatocytes.

Finally, we demonstrated that KLF-10 partly inhibits the glucose induction of ChREBP target gene expression. Consistent with our data, another group reported that the deletion of *KLF-10* causes increased expression of ChREBP target genes in the mouse liver (Fig. 3). Moreover, KLF-10 inhibits *Chrebp* expression in rat hepatocytes (Fig. 3). A similar feedback system is also observed between PER2 and CLOCK/BMAL1. The CLOCK/BMAL1 heterodimers recognize the perfect E-box in the ChoRE of the *PER2* promoter region and induce *Per2* mRNA expression [6]. In turn, PER2 represses *CLOCK/BMAL1* expression. Thus, a negative feedback loop between PER2 and CLOCK/BMAL1 is involved in the regulation of circadian rhythm [6]. Similarly, we had previously reported that a feedback loop similar to this is evident between *Chrebp* and *Bhlhb2*. Thus, these results indicate that a negative feedback loop between ChREBP and KLF-10 operates in the regulation of *de novo* lipogenesis and that KLF-10 plays an important role in preventing overshoot of ChREBP-mediated lipid synthesis (Fig. 4).

In conclusion, we demonstrated that *KLF-10* expression is regulated by the glucose-activated transcription factor, ChREBP, which in turn, inhibits glucose-induced ChREBP target gene expression. Our data suggest that the negative feedback loop between ChREBP and transcriptional repressors such as KLF-10 and BHLHB2 is involved in the regulation of hepatic lipogenesis. To identify the feedback loop system between ChREBP and transcriptional repressors in future will be helpful to better understand the mechanisms underlying the regulation of lipogenesis and the development of treatments for metabolic syndrome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.016.

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Short-term effects of liraglutide on visceral fat adiposity, appetite, and food preference: a pilot study of obese Japanese patients with type 2 diabetes

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Abstract

Background: To examine the effects of liraglutide, a glucagon-like peptide-1 (GLP-1) analogue, on visceral fat adiposity, appetite, food preference, and biomarkers of cardiovascular system in Japanese patients with type 2 diabetes.

Methods: The study subjects were 20 inpatients with type 2 diabetes treated with liraglutide [age; 61.2 ± 14.0 years, duration of diabetes; 16.9 ± 6.6 years, glycated hemoglobin (HbA1c); 9.1 ± 1.2%, body mass index (BMI); 28.3 ± 5.2 kg/m^2, mean ± SD]. After improvement in glycemic control by insulin or oral glucose-lowering agents, patients were switched to liraglutide. We assessed the estimated visceral fat area (eVFA) by abdominal bioelectrical impedance analysis, glycemic control by the 75-g oral glucose tolerance test (OGTT) and eating behavior by the Japan Society for the Study of Obesity questionnaire.

Results: Treatment with liraglutide (dose range: 0.3 to 0.9 mg/day) for 20.0 ± 6.4 days significantly reduced waist circumference, waist/hip ratio, eVFA. It also significantly improved the scores of eating behavior, food preference and the urge for fat intake and tended to reduce scores for sense of hunger. Liraglutide increased serum C-peptide immunoreactivity and disposition index.

Conclusions: Short-term treatment with liraglutide improved visceral fat adiposity, appetite, food preference and the urge for fat intake in obese Japanese patients with type 2 diabetes.

Keywords: liraglutide, glucagon-like peptide-1, obesity, eating behavior

Introduction

The prevalence of type 2 diabetes has rapidly increased worldwide [1] including Western and Asian countries [2]. Type 2 diabetes is a major risk for cardiovascular events. Obesity, especially visceral fat adiposity, also increases the risk of type 2 diabetes, hypertension, dyslipidemia, and atherosclerosis, suggesting that obese patients with type 2 diabetes are at high risk for cardiovascular diseases [3]. The World Health Organization (WHO) projects that 2.3 billion adults are overweight and > 700 million are obese [4]. The association between type 2 diabetes and overweight/obesity is indisputable. In this sense, it is necessary to develop effective and efficient therapeutic strategy for obese type 2 diabetes. However, the treatment for obese type 2 diabetes often encounters difficulties with regard to the control of weight and appetite. Moreover, treatment with insulin, sulfonylurea, and thiazolidinedione increase appetite and body weight, frequently resulting in poor glycemic control.

Dietary fat intake alters glucose and lipid metabolism [5] and correlates with cardiovascular risk in type 2 diabetes [6]. The intake of animal fat, especially saturated fat, is considered to be associated with type 2 diabetes and cardiovascular diseases [7]. Higher intake of polyunsaturated fat

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relative to saturated fat correlates negatively with the incidence of type 2 diabetes [8,9]. In addition to calorie restriction, the control of food preference is also another important factor in the treatment of obese type 2 diabetes.

Liraglutide is a glucagon-like peptide-1 (GLP-1) analogue with 97% structural homology to human GLP-1. Native GLP-1 has a short elimination half-life of 1-2 min, whereas liraglutide has a long half-life of about 13 hours and can be administered once a day [10]. GLP-1 is a naturally occurring incretin hormone with a potent blood-glucose lowering action only during hyperglycemia because it induces insulin secretion and reduces glucagon secretion in a glucose-dependent manner [11]. In addition, GLP-1 delays gastric emptying and induces satiety, leading to decreased energy intake and weight reduction. The underlying mechanisms of weight loss are most probably a combination of the effects of GLP-1 on the gastrointestinal tract and the brain [12]. Recent experiments show that the anorectic effect of peripheral GLP-1 administration is mediated both by the activation of GLP-1 receptor (GLP-1R) expressed on vagal afferents and by the GLP-1R activation in central nervous system [13]. GLP-1 also has various extrapancreatic actions such as the cardiovascular system [4] and is considered a promising new agent for the treatment of type 2 diabetes and cardiovascular diseases linked to obesity-type 2 diabetes.

In the present study, we investigated the effects of liraglutide on visceral fat adiposity, eating behavior, and cardiovascular biomarkers in controlled hospitalized patients with type 2 diabetes.

Materials and methods

Subjects

All enrolled subjects were hospitalized in the Division of Endocrinology & Metabolism, Osaka University Hospital. They represented inpatients with type 2 diabetes treated for the first time with liraglutide between August 2010 and April 2011. Upon admission, each patient was treated with insulin or oral glucose-lowering agents under diet therapy to improve plasma glucose levels. After achieving the target levels of glycemic control [fasting plasma glucose (FPG) < 150 mg/dL and postprandial 2-h plasma glucose < 200 mg/dL], treatment with insulin or oral glucose-lowering agents was replaced with liraglutide at 0.3 mg/day, which was increased by 0.3 mg/day every one week to a final dose of 0.9 mg/day, representing the maximum dose in Japan. Introduction of liraglutide was decided by each attending physician after confirmation of preserved β-cell function by the examination of fasting serum C-peptide, daily urine C-peptide, and/or, glucagon challenge test. Among the 20 study subjects, liraglutide treatment failed to lower FPG to the target levels even at 0.9 mg/day in 8 patients (sulfonylurea (SU), n = 4; biguanide (BG), n = 1; SU+BG, n = 3), necessitating the addition of oral glucose-lowering agents according to the assessment of the attending physician. Written consent was obtained from each subject after explaining the purpose and complications of the study. The study protocol was approved by the human ethics committee of Osaka University and was registered with the University hospital Medical Information Network (Number: UMIN 000004192).

Clinical examination

Various metabolic parameters were measured on admission, liraglutide induction, and discharge. Waist circumference at umbilical level was measured in standing position with a non-stretchable tape in the late phase of expiration. Estimated visceral fat area (eVFA) was measured by abdominal bioelectrical impedance analysis (BIA), as described previously [14].

Several diabetic parameters were examined before and after liraglutide use. Each subject received a 75-g oral glucose tolerance test (OGTT) after 10- to 12-h overnight fast. Insulin secretion was evaluated by fasting serum C-peptide (F-sCPR), C-peptide index (CPI), insulinogenic index, and disposition index. Insulin resistance was assessed by homeostasis model assessment of insulin resistance (HOMA-IR) and the Matsuda index. These parameters were calculated by the following formula: CPI = F-sCPR (ng/mL) × 100/fasting glucose (mg/dL), insulinogenic index = [(insulin at 30 min) - (insulin at 0 min)]/[(glucose at 30 min) - (glucose at 0 min)], HOMA-IR = fasting glucose (mg/dL) × fasting insulin (μU/ml)/405, composite (Matsuda) index and disposition index were calculated using the MINMOD Millenium software [15,16]. High-sensitivity C-reactive protein (hsCRP) (N-Latex CRP II, Dade Behring Inc, Marburg, Germany) and soluble intercellular adhesion molecule-1 (sICAM-1) (Pierce Biotechnology, Rockford, IL) were also measured before and after liraglutide treatment.

To evaluate dietary intake, staple food and non-staple food were quantified at each meal by the nurses. Every intake of staple food and non-staple food was rated on an eleven-point scale ranging from 0 (no intake) to 10 (full intake), respectively. For example, if all staple food was consumed every meal, the daily score for staple food intake was 30 point/day. Scores were recalculated based on previous prescribed total daily calorie when the diet was changed. Food intake was quantified by the daily average intake from admission to liraglutide induction, and from liraglutide induction to discharge, respectively.

Questionnaire for eating behavior

Eating behavior was assessed by using the questionnaire of The Guideline For Obesity issued by the Japan Society for the Study of Obesity (advocated by Prof. Hironobu

Yoshimatsu), at admission, pre- and post-liraglutide treatment. This questionnaire consists of 55-item questions of seven major scales as follows: 1. Recognition for weight and constitution (e.g., 'Do you think it is easier for you to gain weight than others?', 'Do you think you gain weight because of less exercise?'), 2. External eating behavior (e.g., 'If food smells and looks good, do you eat more than usual?', 'If you walk past the supermarket, do you have the desire to buy something delicious?', 'If you see others eating, do you also have the desire to eat?'), 3. Emotional eating behavior (e.g., 'Do you have the desire to eat when you are irritated?', 'Do you have a desire to eat when you have nothing to do?'), 4. Sense of hunger (e.g. 'Do you get irritated when you feel hungry?', 'Do you often regret because you have eaten a lot of food?'), 5. Eating style (e.g., 'Do you eat fast?', 'Are you known to eat a lot of food?'), 6. Food preference (e.g., 'Do you often eat snack bread?', 'Do you like meat?', 'Do you like noodles?'), 7. Regularity of eating habits (e.g., 'Is your dinner time too late at night?', 'Do you gain body weight during holidays?'). All items were rated on a four-point scale ranging from 1 (seldom) to 4 (very often).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). All analyses were performed using JMP software (JMP 8.0; SAS Institute Inc., Cary, NC). For comparison of results obtained at admission, before and after liraglutide treatment, the Student's t-test was used.

Results

Characteristics of participants

Table 1 shows baseline characteristics of 20 subjects at admission. The mean age was 61.2 years, body mass index (BMI) 28.3 kg/m^2, and the duration of diabetes was 16.9 years. The mean glycated hemoglobin (HbA1c) was 9.1%. At baseline, 65% of patients were treated with insulin (average dosage: 37.3 units/day, maximum dosage: 70 units/day). These parameters indicate that the enrolled patients had relatively long duration of diabetes, mild obesity, high frequency of insulin use, and high dosage of insulin.

After admission, patients were started on insulin or oral glucose-lowering agents under diet therapy until glycemic control reached target levels, considered as relief from gluco-toxicity, and were then introduced to liraglutide treatment at 0.3 mg/day. The dose was gradually increased according to blood glucose levels as described in the Methods section. The number of patients treated with liraglutide at 0.3, 0.6, and 0.9 mg/day at discharge was 1, 9, and 10 patients, respectively. The time period from admission to induction of liraglutide was 11.1 ± 4.7 days and from induction to discharge was 20.0 ± 6.4 days.

Table 1 Baseline characteristics

Sex (Male/Female)	8/12
Age (years)	61.2 ± 14.0
Duration of diabetes (years)	16.9 ± 6.6
HbA1c (%)	9.1 ± 1.2
Body mass index (kg/m^2)	28.3 ± 5.2
Waist circumference (cm)	100.0 ± 11.8
Waist-to-hip ratio	1.01 ± 0.07
Estimated visceral fat area (cm^2)	182.6 ± 68.3
LDL-C (mg/dL)	112.7 ± 29.1
HDL-C (mg/dL)	45.7 ± 8.8
Triglycerides (mg/dL)	141.6 ± 68.2
Uric acid (mg/dL)	6.0 ± 1.5
History of smoking (%)	30
Hypertension (%)	70
Dyslipidemia (%)	95
History of cardiovascular disease (%)	30
Medication for Diabetes	
Biguanide (%)	50
Sulfonylurea (%)	45
Alpha-glucosidase inhibitor (%)	30
Thiazolidinedione (%)	25
DPP-IV inhibitor (%)	10
Glinide (%)	10
Insulin (%)	65

Data are mean ± SD or number of subjects. LDL-C; low-density lipoprotein-cholesterol, HDL-C; high-density lipoprotein-cholesterol, DPP-IV; dipeptidyl peptidase-IV.

Changes in physical parameters and visceral fat adiposity

BMI was significantly reduced by 0.54 ± 0.11 kg/m^2 from admission to liraglutide induction with further significant decrease by 0.93 ± 0.12 kg/m^2 from liraglutide induction to discharge (Figure 1A). The waist circumference decreased by -0.30 ± 0.44 cm from admission to liraglutide induction, and liraglutide treatment significantly decreased waist circumferences by -4.28 ± 0.55 cm from liraglutide induction to discharge (Figure 1B). The waist/hip (W/H) ratio at admission, liraglutide induction, and discharge was 1.01 ± 0.07, 1.00 ± 0.06, and 0.97 ± 0.06, respectively, and was significantly reduced by liraglutide (Figure 1C). The eVFA at admission, liraglutide induction, and discharge were 182.6 ± 68.3 cm^2, 178.1 ± 56.8 cm^2, and 153.4 ± 51.9 cm^2, respectively, (Figure 1D). Similar to waist circumference and W/H ratio, liraglutide significantly reduced eVFA compared with no significant change before liraglutide induction.

Assessment of food intake, appetite, and food preferences

Next, the effects of liraglutide on food intake, appetite, and food preference were evaluated. Liraglutide significantly decreased daily staple food intake (Figure 2A), but did not change significantly the daily intake for

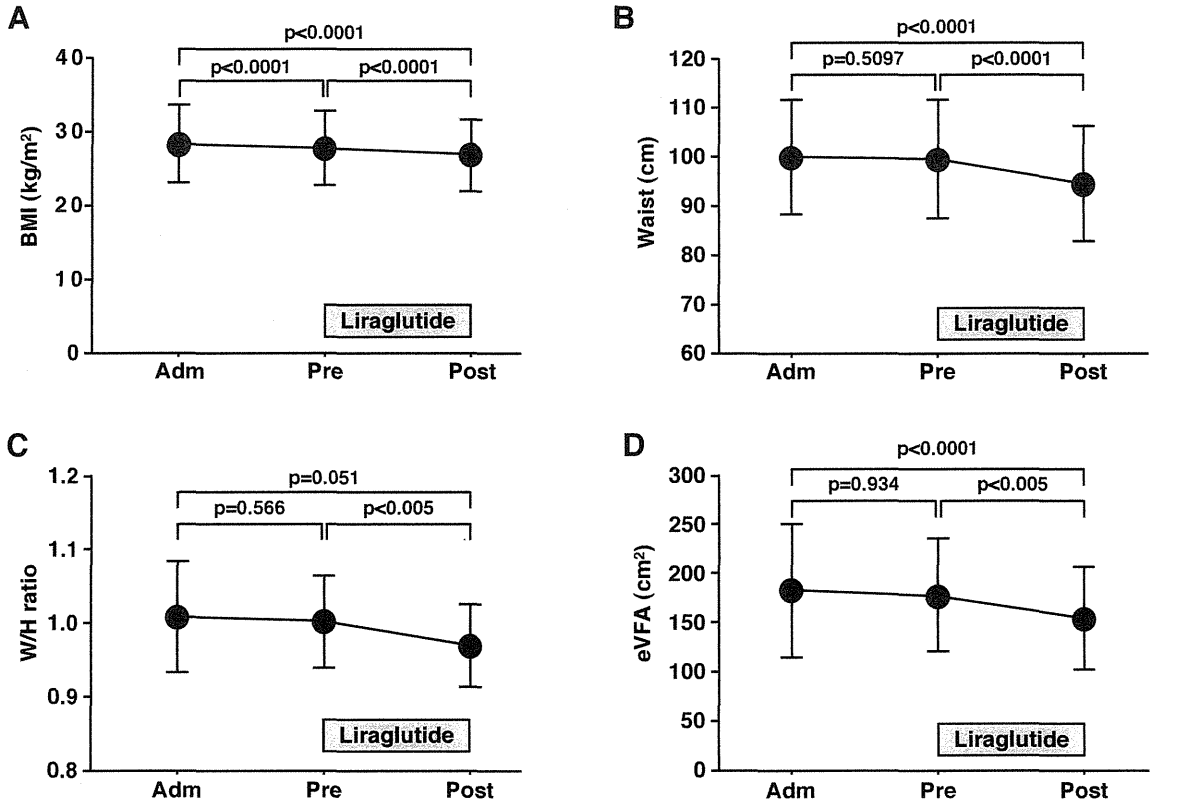

Figure 1 Liraglutide-induced changes in visceral fat adiposity. Data are mean ± SD of 20 patients. BMI, body mass index; W/H, waist/hip; eVFA, estimated visceral fat area; Adm, admission; Pre, pre-liraglutide treatment; Post, post-liraglutide treatment.

non-staple food (Figure 2B). Patients received a questionnaire on eating behavior at admission, pre- and post-liraglutide as described in the Methods section. Figure 2C to 2E show changes in the scores for representative question headings; external eating behavior, sense of hunger, and food preference. These score did not change under conventional glucose-lowering treatment, i.e., before liraglutide induction. However, treatment with liraglutide significantly decreased the scores for external eating behavior (Figure 2C) and tended to decrease the sense of hunger (Figure 2D). The number of patients who reported feeling full stomach was higher after liraglutide induction. Interestingly, liraglutide administration also decreased the score of food preference, related to the obesity phenotype (Figure 2E), and especially reduced the need for fat intake such as fatty foods, meat diet, and snack breads (Figure 2F).

Effect of liraglutide treatment on glucose tolerance

OGTT was conducted just before liraglutide induction, i.e., after relief of gluco-toxicity. Table 2 lists various indices related to insulin sensitivity and insulin secretion measured before and after induction of liraglutide treatment. There were no significant differences in HOMA-IR and the Matsuda index between pre- and post-liraglutide, while fasting serum C-peptide immunoreactivity (F-sCPR) and C-peptide index (CPI) were significantly elevated by liraglutide treatment. Liraglutide also increased the insulinogenic index and disposition index. These data indicate that liraglutide ameliorates insulin secretion as expected.

Effect of liraglutide treatment on biomarkers of cardiovascular system

We also tested the effects of short-term liraglutide treatment on biomarkers of cardiovascular system. Serum hsCRP levels were significantly reduced by liraglutide (1.10 ± 1.03 mg/L at liraglutide induction versus 0.70 ± 0.47 mg/L at discharge, $P < 0.05$). Treatment with liraglutide tended to decrease plasma concentrations of sICAM-1 (198.23 ± 84.95 ng/mL at liraglutide induction versus 180.99 ± 59.27 ng/mL at discharge, $P = 0.067$).

Discussion

The present study demonstrated that short-term liraglutide treatment significantly affected visceral fat adiposity,

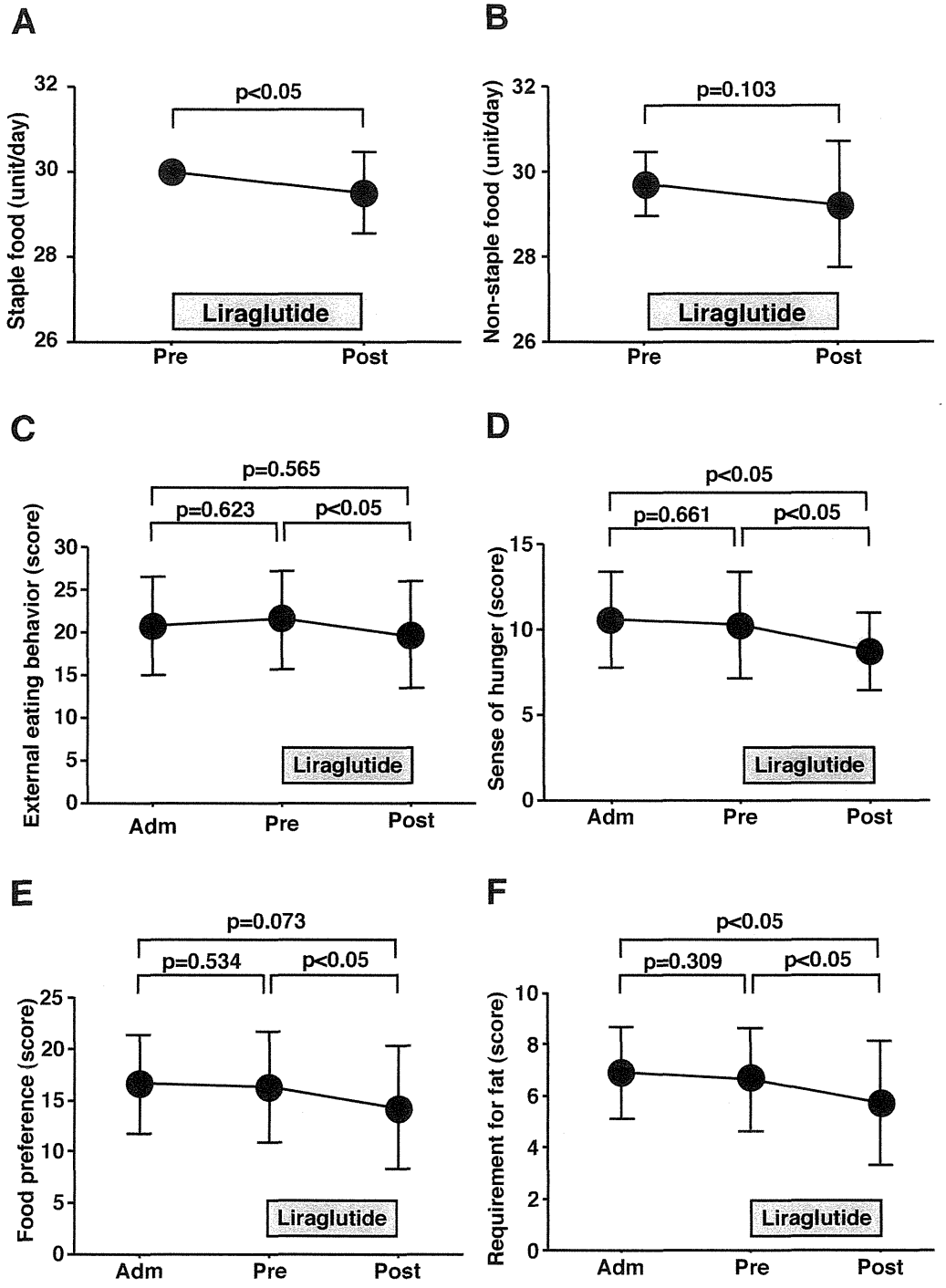

Figure 2 Liraglutide-induced changes in food intake and appetite. Every intake of staple food and non-staple food was rated on an eleven-point scale ranging from 0 (no intake) to 10 (full intake), respectively. Eating behavior was assessed by using a questionnaire as described in method section. Data are mean ± SD of 20 patients. For abbreviation; see Figure 1.

Table 2 Liraglutide-induced changes in parameters relating to glucose metabolism

	Before treatment with liraglutide	After treatment with liraglutide	P value
sCPR(ng/mL)	1.87 ± 1.00	3.25 ± 1.19	< 0.0001
C-peptide Index	1.47 ± 0.75	2.76 ± 0.97	< 0.0001
HOMA-IR	3.52 ± 2.63	4.51 ± 3.27	0.468
Matsuda Index	3.09 ± 2.24	3.16 ± 1.99	0.820
Insulinogenic Index	0.18 ± 0.16	0.32 ± 0.30	0.058
Disposition Index	0.43 ± 0.27	0.76 ± 0.51	< 0.05

Data are mean ± SD. sCPR; serum C-peptide immunoreactivity, HOMA-IR; homeostasis model assessment of insulin resistance.

appetite, food preference, and cardiovascular biomarkers in Japanese patients with type 2 diabetes.

The LEAD-2 trial showed that liraglutide at 1.2 and 1.8 mg/day significantly decreased waist circumference and eVFA compared to the 4 mg/day glimepiride after 26 weeks from introduction, while there was no significant difference in the reductions of waist circumference and eVFA between liraglutide group and placebo group [17]. In the LEAD-3 substudy, a long-term liraglutide monotherapy significantly reduced body weight and fat mass from baseline while body weight and fat mass were increased in the glimepiride group [18]. In the present study, waist circumference, W/H ratio, and eVFA did not change while patients were treated with insulin and/or oral glucose-lowering agents (Figure 1). However, treatment with liraglutide significantly reduced these physical parameters. Furthermore, the daily reduction of waist circumferences was significantly larger during liraglutide treatment than before liraglutide introduction, suggesting that liraglutide effectively decreased visceral fat adiposity. Previous studies reported the lack of GLP-1 receptor expression on adipocytes [19], but recent work has demonstrated GLP-1 receptor expression on adipocytes [20]. Although GLP-1 action and GLP-1 receptor signaling in adipocytes have not been fully elucidated, the present study demonstrated that liraglutide reduced visceral fat adiposity in a direct or indirect fashion. The results also point to a possible anti-atherosclerotic effect for liraglutide partly through reduction of eVFA. Treatment with exenatide, another GLP-1 receptor agonist, exhibited a significant weight reduction when used for 3 years [21]. Previous study showed that body weight did not change following the use of liraglutide by Japanese type 2 diabetes patients, but the mean BMI of those patients was only 23.9 kg/m^2 [22]. As shown in Table 1 the mean BMI of our patients was 28.3 kg/m^2, suggesting that the effect of liraglutide on weight reduction is limited to obese type 2 diabetics.

Several studies have demonstrated that GLP-1 promotes satiety and suppresses energy intake both in animals [23,24] and human subjects [25-28], however, the effect of liraglutide on eating behavior has not been examined in human type 2 diabetics. The present study demonstrated that liraglutide reduced food intake and changed external eating behavior and food preference. Liraglutide significantly reduced the intake for staple food, e.g., rice and/or bread. Interestingly, liraglutide significantly decreased the scores for external eating behavior and food preference (Figure 2C and 2E). Questions on external eating behavior evaluate appetite increase through sight and smell senses. Questions about food preference measure foods directly associated with obesity. Such improvement in eating behavior induced by liraglutide has not been reported for other glucose-lowering agents. Furthermore, liraglutide significantly reduced the urge for fat intake (Figure 2F). Although the findings of epidemiological studies on the association of total dietary fat with type 2 diabetes have been inconsistent [29-32], dietary fat intake impairs glucose metabolism [5] and is strongly related to cardiovascular risk in type 2 diabetes [6]. A high intake of saturated fat is considered to associate with type 2 diabetes and cardiovascular diseases [7]. In this sense, liraglutide may be beneficial in reducing fat preference, related to obese type 2 diabetes, although the long-term effects of liraglutide on eating behavior remain to be elucidated. It would be better to refine the questions on food preference to identify the types of fat.

GLP-1 receptor agonist exerts glucose-lowering effect mainly by stimulating glucose-mediated secretion of insulin from β-cells. Insulin secretion is often deteriorated in various degrees in Japanese and the other Asian patients with type 2 diabetes compared to Caucasian [2], and thus the effect of liragulutide on insulin secretion is worth evaluating in Japanese type 2 diabetes subjects. As shown in Table 2 liraglutide treatment increased sCPR and C-peptide Index, indicating that insulin secretion was enhanced by liraglutide. Such changes may appear the increases of HOMA-IR and Matsuda Index, suggesting that these indices may not exactly reflect insulin sensitivity under liraglutide treatment. There is a possibility that long-term liraglutide treatment may ameliorate insulin resistance by weight reduction through decrease of appetite. Further clinical investigations may be needed in future.

The extrapancreatic actions of GLP-1 have been demonstrated, especially the beneficial its effect on the cardiovascular system [4]. GLP-1 has a protective action