

【3】研究成果の刊行物・別刷

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

Yukio Horikawa, Mayumi Enya, Katsumi Iizuka, Gui Ying Chen, Shin-ichi Kawachi, Tetsuya Suwa, Jun Takeda*

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 events¹, 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) secretion². 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.

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 ($\Delta\text{AUC}_{0-2\text{ h}}$) 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)

Department of Diabetes and Endocrinology, Graduate School of Medicine, Gifu University, Gifu, Japan

*Corresponding author: Jun Takeda Tel: +81-58-230-6371 Fax: +81-58-230-6376

E-mail address: jtakeda@gifu-u.ac.jp

Received 27 July 2010; revised 15 September 2010; accepted 29 September 2010

was given 0.5 h before oral loading. Test doses of α -GIs were determined from the ED₅₀ 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 \pm 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 Δ AUC_{0-2h} 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 Δ AUC_{0-2h} 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).

DISCUSSION

Recently, suppression of postprandial hyperglycemia has come to be considered important for prevention of atherosclerosis³. Repeated episodes of blood glucose fluctuation accelerate the adhesion of monocytes to vascular endothelial cells and enhance the development/progression of atherosclerosis⁸. 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

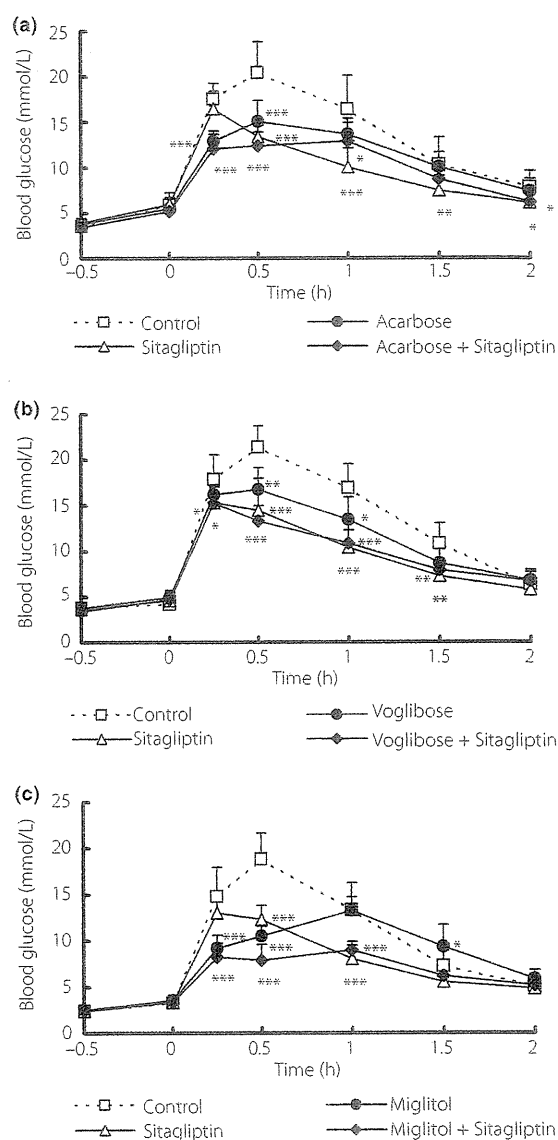


Figure 1 | Blood glucose profiles after oral administration of α -glucosidase inhibitor (α -GI) and sitagliptin (0.3 mg/kg) in maltose-loaded normal mice. (a) Acarbose, 10 mg/kg; (b) voglibose, 0.1 mg/kg; (c) miglitol, 3 mg/kg. Each value represents mean \pm SD of 8–10 mice. **P* < 0.05, ***P* < 0.01,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

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 -cells⁹. 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 Δ 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 \pm 4.6 ^a	–	15.3 \pm 2.6 ^a	111.9 \pm 44.8 ^{ab}	2.18 \pm 0.90 ^a
Acarbose, 10 mg/kg	15.6 \pm 2.6 ^b	25.4	10.0 \pm 2.5 ^b	89.5 \pm 25.8 ^a	2.37 \pm 0.76 ^a
Sitagliptin, 0.3 mg/kg	12.3 \pm 3.3 ^b	41.1	10.9 \pm 1.7 ^b	173.9 \pm 53.4 ^b	3.33 \pm 1.44 ^a
Combination	13.8 \pm 2.3 ^b	34.0	8.5 \pm 2.3 ^b	118.8 \pm 68.9 ^{ab}	10.76 \pm 6.34 ^b
Control	20.9 \pm 3.2 ^a	–	17.2 \pm 2.4 ^a	113.7 \pm 48.2 ^a	2.23 \pm 1.86 ^a
Voglibose, 0.1 mg/kg	16.1 \pm 3.2 ^b	23.0	12.3 \pm 1.7 ^b	111.9 \pm 17.2 ^a	2.78 \pm 1.23 ^a
Sitagliptin, 0.3 mg/kg	12.7 \pm 2.9 ^b	39.2	11.6 \pm 2.4 ^b	229.0 \pm 93.0 ^b	3.54 \pm 2.33 ^{ac}
Combination	13.6 \pm 1.3 ^b	34.9	10.7 \pm 1.8 ^b	142.9 \pm 43.1 ^a	5.52 \pm 2.34 ^{bc}
Control	17.9 \pm 3.6 ^a	–	15.5 \pm 2.6 ^a	158.9 \pm 44.8 ^a	2.37 \pm 1.33 ^a
Miglitol, 3 mg/kg	14.4 \pm 1.7 ^b	19.6	9.7 \pm 1.3 ^b	96.4 \pm 18.9 ^a	3.29 \pm 1.13 ^a
Sitagliptin, 0.3 mg/kg	11.5 \pm 1.6 ^{bc}	35.8	10.0 \pm 1.8 ^b	252.5 \pm 86.1 ^b	4.62 \pm 3.06 ^a
Combination	9.4 \pm 1.3 ^c	47.5	6.2 \pm 1.3 ^c	107.3 \pm 20.7 ^a	11.22 \pm 9.72 ^b

Maltose-loading tests used for normal mice. Enteral nutrition (EN)-loading tests used for mice fed high-fat diet for 6 weeks. Each value represents the mean \pm 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 reported¹⁰. Because the CRP elevation is related to coronary heart disease, stroke and mortality⁵, 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 \pm 84.4 pmol/L; high-fat control group: 253.1 \pm 72.3 pmol/L, $P < 0.05$ vs normal diet group; miglitol alone group: 189.4 \pm 110.2 pmol/L; sitagliptin alone group: 359.9 \pm 187.7 pmol/L; combination group: 161.9 \pm 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 appetite¹², 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 tract¹³, 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.

ACKNOWLEDGEMENTS

We thank H Tsuchida and K Yokoyama for technical assistance. This work was supported by a Health and Labor Science Research Grant from the Japanese Ministry of Health, Labor and Welfare, a KAKENHI, Grant-in-Aid for Scientific Research from the Japanese Ministry of Science, Education, Sports, Culture and Technology, and a New Energy and Industrial Technology Development Organization Grant. The authors report no conflicts of interest.

REFERENCES

- Chiasson JL, Josse RG, Gomis R, *et al.* Stop-NIDDM Trial Research Group. Acarbose prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. *Lancet* 2002; 359: 2072–2077.
- Narita T, Katsuura Y, Sato T, *et al.* Miglitol induces prolonged and enhanced glucagons-like peptide-1 and reduced gastric inhibitory polypeptide responses after ingestion of a mixed meal in Japanese Type 2 diabetic patients. *Diabet Med* 2009; 26: 187–188.
- Esposito K, Ciotola M, Carleo D, *et al.* Post-meal glucose peaks at home associate with carotid intima-media thickness in type 2 diabetes. *J Clin Endocrinol Metab* 2008; 93: 1345–1350.

4. Arakawa M, Mita T, Azuma K, *et al.* Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. *Diabetes* 2010; 59: 1030–1037.
5. Kaptoge S, Di Angelantonio E, Lowe G, *et al.* C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* 2010; 375: 132–140.
6. Yamazaki K, Inoue T, Yasuda N, *et al.* Comparison of efficacies of a dipeptidyl peptidase IV inhibitor and alpha-glucosidase inhibitors in oral carbohydrate and meal tolerance tests and the effects of their combination in mice. *J Pharmacol Sci* 2007; 104: 29–38.
7. Moritoh Y, Takeuchi K, Hazama M. Combination treatment with alogliptin and voglibose increases active GLP-1 circulation, prevents the development of diabetes and preserves pancreatic beta-cells in prediabetic db/db mice. *Diabetes Obes Metab* 2010; 12: 224–233.
8. Mita T, Otsuka A, Azuma K, *et al.* Swings in blood glucose levels accelerate atherogenesis in apolipoprotein E-deficient mice. *Biochem Biophys Res Commun* 2007; 358: 679–685.
9. Fukaya N, Mochizuki K, Tanaka Y, *et al.* The alpha-glucosidase inhibitor miglitol delays the development of diabetes and dysfunctional insulin secretion in pancreatic beta-cells in OLETF rats. *Eur J Pharmacol* 2009; 624: 51–57.
10. McLaughlin T, Abbasi F, Lamendola C, *et al.* Differentiation between obesity and insulin resistance in the association with C-reactive protein. *Circulation* 2002; 106: 2908–2912.
11. Lee A, Patrick P, Wishart J, *et al.* The effects of miglitol on glucagons-like peptide-1 secretion and appetite sensations in obese type 2 diabetics. *Diabetes Obes Metab* 2002; 4: 329–335.
12. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006; 3: 153–165.
13. Qualmann C, Nauck MA, Holst JJ, *et al.* Glucagon-like peptide 1 (7-36 amide) secretion in response to luminal sucrose from the upper and lower gut. A study using alpha-glucosidase inhibition (acarbose). *Scand J Gastroenterol* 1995; 30: 892–896.



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

Katsumi Iizuka^{a,b}, Jun Takeda^a, Yukio Horikawa^{a,*}

^aDepartment of Diabetes and Endocrinology, Graduate School of Medicine, Gifu University, Gifu 501-1194, Japan

^bGifu University, University Hospital Center for Nutritional Support and Infection Control, Gifu 501-1194, Japan

ARTICLE INFO

Article history:

Received 4 August 2011

Available online 11 August 2011

Keywords:

ChREBP
KLF-10
Lipogenesis
Circadian rhythm
Liver
ChoRE

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 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.

© 2011 Elsevier Inc. All rights reserved.

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*

* Corresponding author. Fax: +81 58 230 6376.

E-mail address: yhorikaw@gifu-u.ac.jp (Y. Horikawa).

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 manufacturer'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 μ l of Lipofectamine[®] 2000 transfection reagent, 1.4 μ g of the series of pGL3-KLF-10, and 0.1 μ g 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 manufacturer's protocol. To determine the glucose dependency of the glucose response region in rat *Klf-10* promoter, cells were transfected with 1.0 μ g of the pGL3 promoter 3xK10-1 or 3xK10-2 putative ChoRE vectors, 0.1 μ g of pGL4 TK RLuc vectors, and 0.4 μ g 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 T₃ and 100 μ g/ml pen/strep for 4 h and then for 2 h in DMEM supplemented with 10 nM T₃ and 100 μ g/ml pen/strep. After the incubations, 10% formaldehyde (270 μ l) 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 μ l, 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'-TCGACCAATCAGCGGTAAGG-3' (Reverse).

2.6. Data presentation and statistical methods

All data are expressed as mean \pm 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

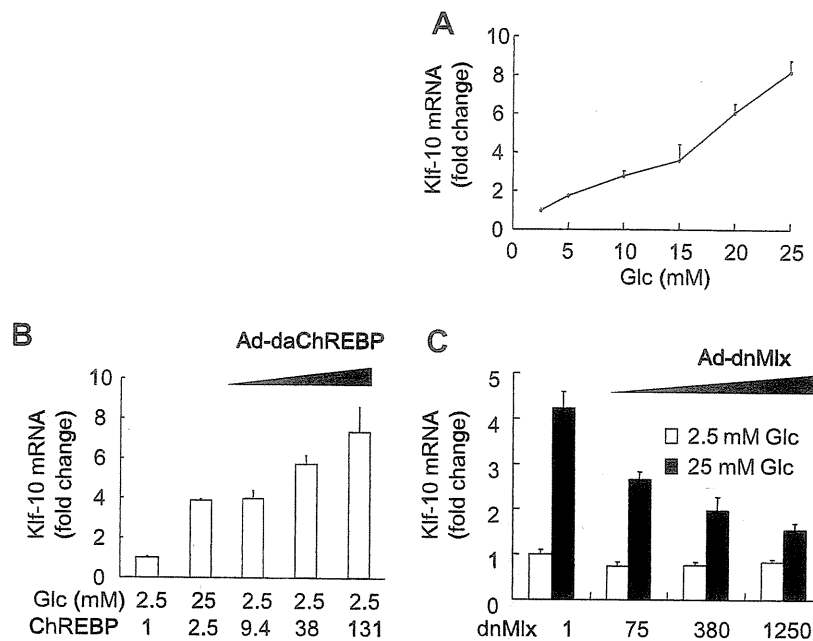


Fig. 1. Regulation of the rat *Klf-10* promoter by glucose (A) Dose-dependent effects of glucose on *Klf-10* mRNA expression in rat primary hepatocytes. Primary hepatocytes isolated from 6-week-old male Wistar rats were cultured for 18 h in culture medium containing 2.5, 5.0, 10, 15, 20 or 25 mM glucose. 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 \pm SD ($n = 3$) of two independent experiments. (C) Adenoviral overexpression of dnMLX inhibits glucose-induced *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 \pm 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 (CAY-GNGN5CNCRTG), 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.

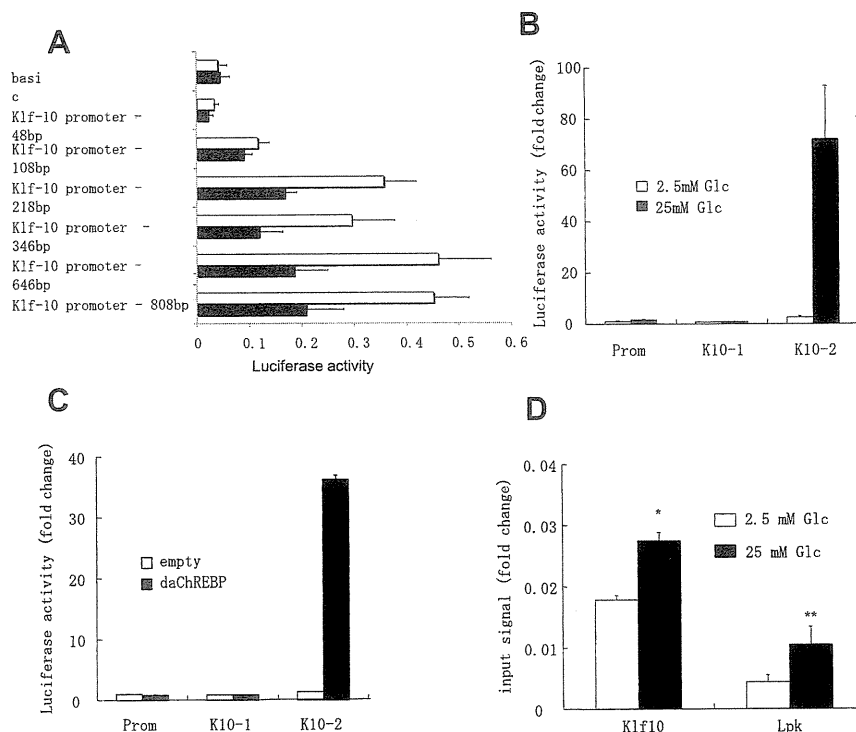


Fig. 2. Direct regulation of the rat *Klf-10* promoter by ChREBP (A) A schematic representation of the rat *Klf-10* promoter luciferase deletion constructs and the effect of these deletions on relative luciferase activity at low (2.5 mM) or high (25 mM) glucose concentrations. A range of reporter gene plasmids (–808, –646, –346, –218, –108, and –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 are represented as mean \pm SD ($n = 6$). The figure represents data from two independent experiments. (B) Putative ChRE in the rat *Klf-10* is a response element activated by glucose and ChREBP. The pGL3 promoter vector containing tandem 3xK10-1 or 3xK10-2 putative ChREs 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 vector. Data are mean \pm SD ($n = 6$) of two independent experiments. (C) Putative ChRE in the rat *Klf-10* is a response element activated by glucose and ChREBP. The pGL3 promoter vector containing tandem 3xK10-1 or 3xK10-2 putative ChREs 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 vector. Data are mean \pm SD ($n = 6$) of two 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 nonspecific rabbit IgG were used as negative controls. Immunoprecipitated samples were subjected to PCR using primers to amplify ChRE-containing regions of the rat *Klf-10* or *Lpk* promoters. All experiments were performed in duplicate.

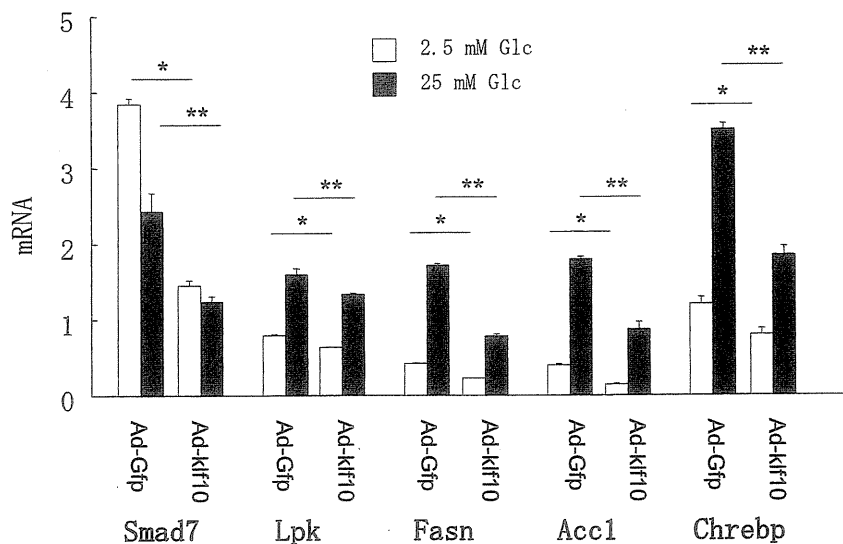


Fig. 3. Adenoviral overexpression of KLF-10 causes decreased ChREBP target gene expression. KLF-10 dose-dependently inhibited the glucose induction of ChREBP target gene expression in rat primary hepatocytes. In hepatocytes infected with 50 m.o.i. of Ad-KLF-10 adenovirus, *Klf10* mRNA expression increased 500-fold. *Smad7*, *Klf-10*, *Fasn*, *Acc1*, *Lpk*, and *Chrebp* mRNA levels were detected by TaqMan[®] RT-PCR and corrected with *Pol2* mRNA. Data are represented as mean \pm 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 have reported that *Chrebp* mRNA is expressed in the lipogenic tissues,

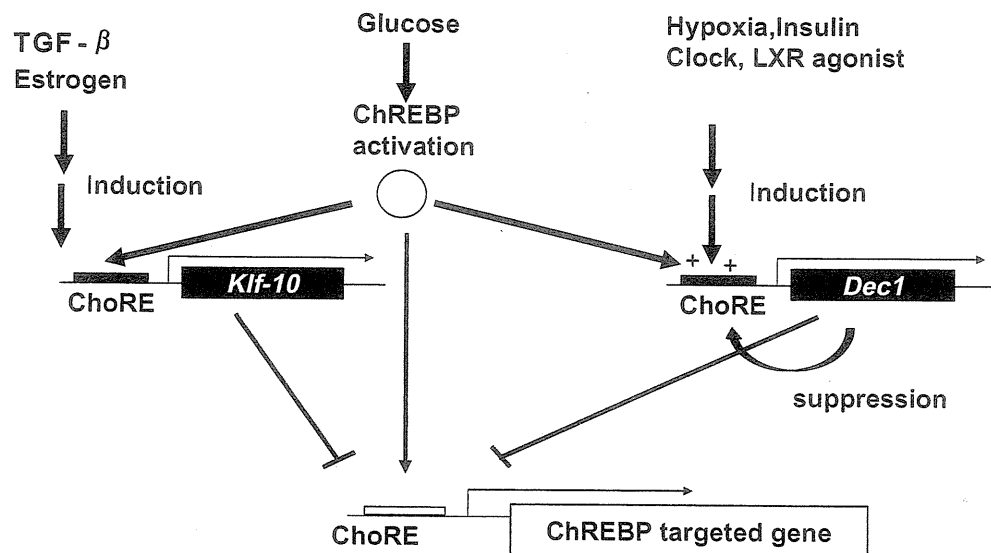


Fig. 4. A schematic representation of the feedback loop model for the *de novo* coordinate regulation of lipogenesis. In rat hepatocytes, ChREBP induces the transcription repressors such as KLF-10 and BHLHB2. In turn, these repressors inhibit the glucose induction of ChREBP target gene expression (*L-pk*,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

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (K. Iizuka), the Kao Research Council for the Study of Healthcare Science (K. Iizuka), and in part by a New Energy and Industrial Technology Development Organization grant (Y. Horikawa).

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.

References

- [1] J.C. Cohen, J.D. Horton, H.H. Hobbs, Human fatty liver disease: old questions and new insights, *Science* 332 (2011) 1519–1523.
- [2] K. Uyeda, J.J. Repa, Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis, *Cell. Metab.* 4 (2006) 107–110.
- [3] H. Yamashita, M. Takenoshita, M. Sakurai, R.K. Bruick, W.J. Henzel, W. Shillinglaw, D. Arnot, K. Uyeda, A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver, *Proc. Natl. Acad. Sci. USA* 98 (2001) 9116–9121.
- [4] K. Iizuka, R.K. Bruick, G. Liang, J.D. Horton, K. Uyeda, Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis, *Proc. Natl. Acad. Sci. USA* 101 (2004) 7281–7286.

- [5] K. Iizuka, Y. Horikawa, ChREBP: a glucose-activated transcription factor involved in the development of metabolic syndrome, *Endocr. J.* 55 (2008) 617–624.
- [6] K. Iizuka, B. Miller, K. Uyeda, Deficiency of carbohydrate-activated transcription factor ChREBP prevents obesity and improves plasma glucose control in leptin-deficient (ob/ob) mice, *Am. J. Physiol. Endocrinol. Metab.* 291 (2006) E358–E364.
- [7] R. Dentin, F. Benhamed, I. Hainault, V. Fauveau, F. Foufelle, J.R. Dyck, J. Girard, C. Postic, Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice, *Diabetes* 55 (2006) 2159–2170.
- [8] B.B. McConnell, V.W. Yang, Mammalian Krüppel-like factors in health and diseases, *Physiol. Rev.* 90 (2010) 1337–1381.
- [9] M.P. Fautsch, A. Vrabel, D. Rickard, M. Subramaniam, T.C. Spelsberg, E.D. Wieben, Characterization of the mouse TGFbeta-inducible early gene (TIEG): conservation of exon and transcriptional regulatory sequences with evidence of additional transcripts, *Mamm. Genome* 9 (1998) 838–842.
- [10] B. Neve, M.E. Fernandez-Zapico, V. Ashkenazi-Katalan, C. Dina, Y.H. Hamid, E. Joly, E. Vaillant, Y. Benmezroua, E. Durand, N. Bakaher, V. Delannoy, M. Vaxillaire, T. Cook, G.M. Dallinga-Thie, H. Jansen, M.A. Charles, K. Clément, P. Galan, S. Hercberg, N. Helbecque, G. Charpentier, M. Prentki, T. Hansen, O. Pedersen, R. Urrutia, D. Melloul, P. Froguel, Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function, *Proc. Natl. Acad. Sci. USA* 102 (2005) 4807–4812.
- [11] E. Kuroda, Y. Horikawa, M. Enya, N. Oda, E. Suzuki, K. Iizuka, J. Takeda, Identification of minimal promoter and genetic variants of Krüppel-like factor 11 gene and association analysis with type 2 diabetes in Japanese, *Endocr. J.* 56 (2009) 275–286.
- [12] F. Guillaumond, A. Gréchez-Cassiau, M. Subramaniam, S. Brangolo, B. Peteri-Brünback, B. Staels, C. Fiévet, T.C. Spelsberg, F. Delaunay, M. Teboul, Krüppel-like factor KLF10 is a link between the circadian clock and metabolism in liver, *Mol. Cell. Biol.* 30 (2010) 3059–3070.
- [13] T. Hirota, T. Okano, K. Kokame, H. Shirogami-Ikejima, T. Miyata, Y. Fukada, Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts, *J. Biol. Chem.* 277 (2002) 44244–44251.
- [14] L. Ma, L.N. Robinson, H.C. Towle, ChREBP**Mlx* is the principal mediator of glucose-induced gene expression in the liver, *J. Biol. Chem.* 281 (2006) 28721–28730.
- [15] S. Honma, T. Kawamoto, Y. Takagi, K. Fujimoto, F. Sato, M. Noshiro, Y. Kato, K. Honma, *Dec1* and *Dec2* are regulators of the mammalian molecular clock, *Nature* 419 (2002) 841–844.
- [16] K. Iizuka, Y. Horikawa, Regulation of lipogenesis via BHLHE22/DEC1 and ChREBP feedback looping, *Biochem. Biophys. Res. Commun.* 374 (2008) 95–100.
- [17] K. Iizuka, J. Takeda, Y. Horikawa, Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes, *FEBS Lett.* 583 (2009) 2882–2886.
- [18] K. Iizuka, J. Takeda, Y. Horikawa, Hepatic overexpression of dominant negative *Mlx* improves metabolic profile in diabetes-prone C57BL/6J mice, *Biochem. Biophys. Res. Commun.* 379 (2009) 499–504.

ORIGINAL INVESTIGATION

Open Access

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

Kana Inoue¹, Norikazu Maeda^{1*}, Susumu Kashine¹, Yuya Fujishima¹, Junji Kozawa¹, Aki Hiuge-Shimizu¹, Kohei Okita¹, Akihisa Imagawa¹, Tohru Funahashi² and Ichihiro Shimomura¹

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); $9.1 \pm 1.2\%$, body mass index (BMI); 28.3 ± 5.2 kg/m², mean \pm 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

* Correspondence: norikazu_maeda@endmet.med.osaka-u.ac.jp

¹Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, 2-2-B5, Yamada-oka, Suita, Osaka 565-0871, Japan
Full list of author information is available at the end of the article

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) \times 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) \times fasting insulin (μ U/ml)/405, composite (Matsuda) index and disposition index were calculated using the MINMOD Millennium 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 \pm 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/m², 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 \pm 4.7 days and from induction to discharge was 20.0 \pm 6.4 days.

Table 1 Baseline characteristics

Sex (Male/Female)	8/12
Age (years)	61.2 \pm 14.0
Duration of diabetes (years)	16.9 \pm 6.6
HbA1c (%)	9.1 \pm 1.2
Body mass index (kg/m ²)	28.3 \pm 5.2
Waist circumference (cm)	100.0 \pm 11.8
Waist-to-hip ratio	1.01 \pm 0.07
Estimated visceral fat area (cm ²)	182.6 \pm 68.3
LDL-C (mg/dL)	112.7 \pm 29.1
HDL-C (mg/dL)	45.7 \pm 8.8
Triglycerides (mg/dL)	141.6 \pm 68.2
Uric acid (mg/dL)	6.0 \pm 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

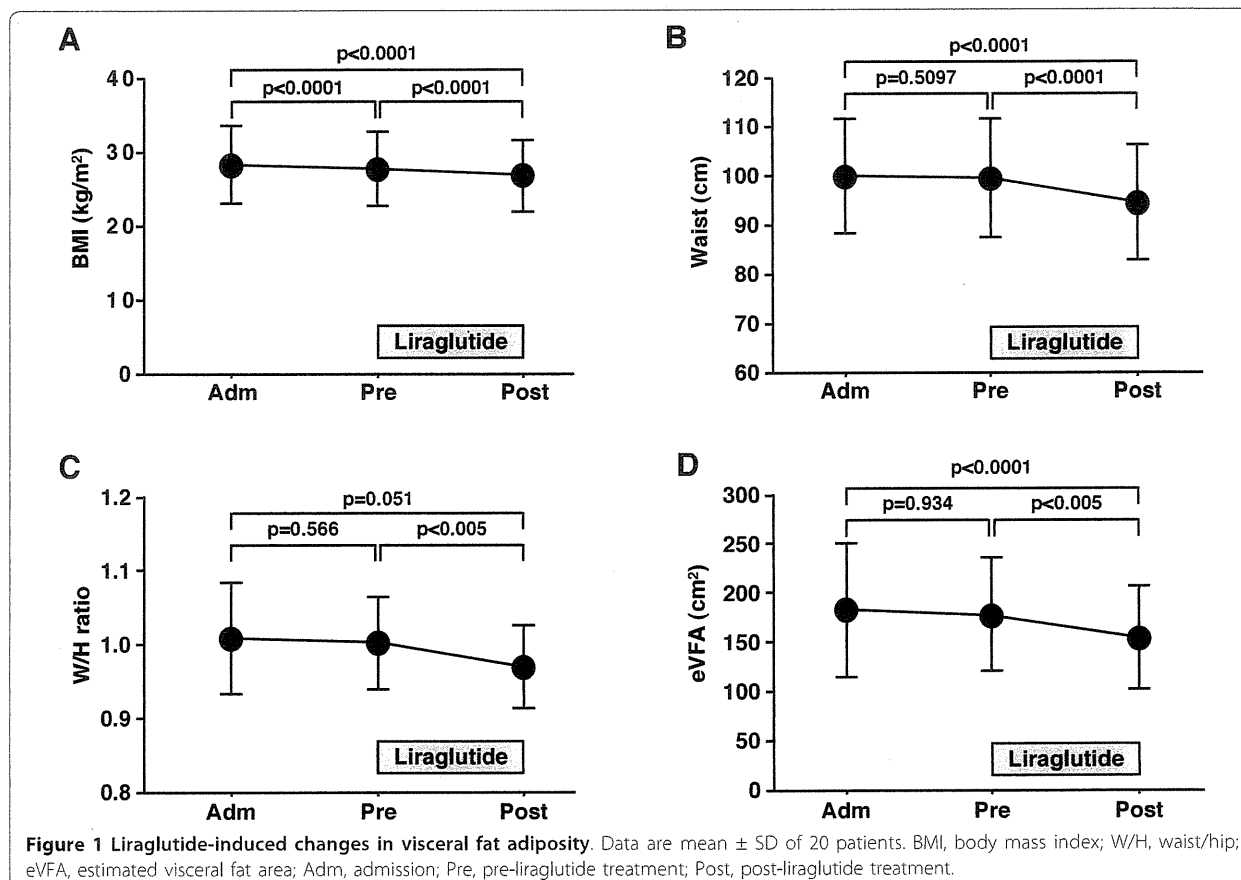
Data are mean \pm SD or number of subjects. LDL-C; low-density lipoprotein-cholesterol, HDL-C; high-density lipoprotein-cholesterol, DPP-IV; dipeptidyl peptidase-IV.

Changes in physical parameters and visceral fat adiposity

BMI was significantly reduced by 0.54 \pm 0.11 kg/m² from admission to liraglutide induction with further significant decrease by 0.93 \pm 0.12 kg/m² from liraglutide induction to discharge (Figure 1A). The waist circumference decreased by -0.30 \pm 0.44 cm from admission to liraglutide induction, and liraglutide treatment significantly decreased waist circumferences by -4.28 \pm 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 \pm 0.07, 1.00 \pm 0.06, and 0.97 \pm 0.06, respectively, and was significantly reduced by liraglutide (Figure 1C). The eVFA at admission, liraglutide induction, and discharge were 182.6 \pm 68.3 cm², 178.1 \pm 56.8 cm², and 153.4 \pm 51.9 cm², 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



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,

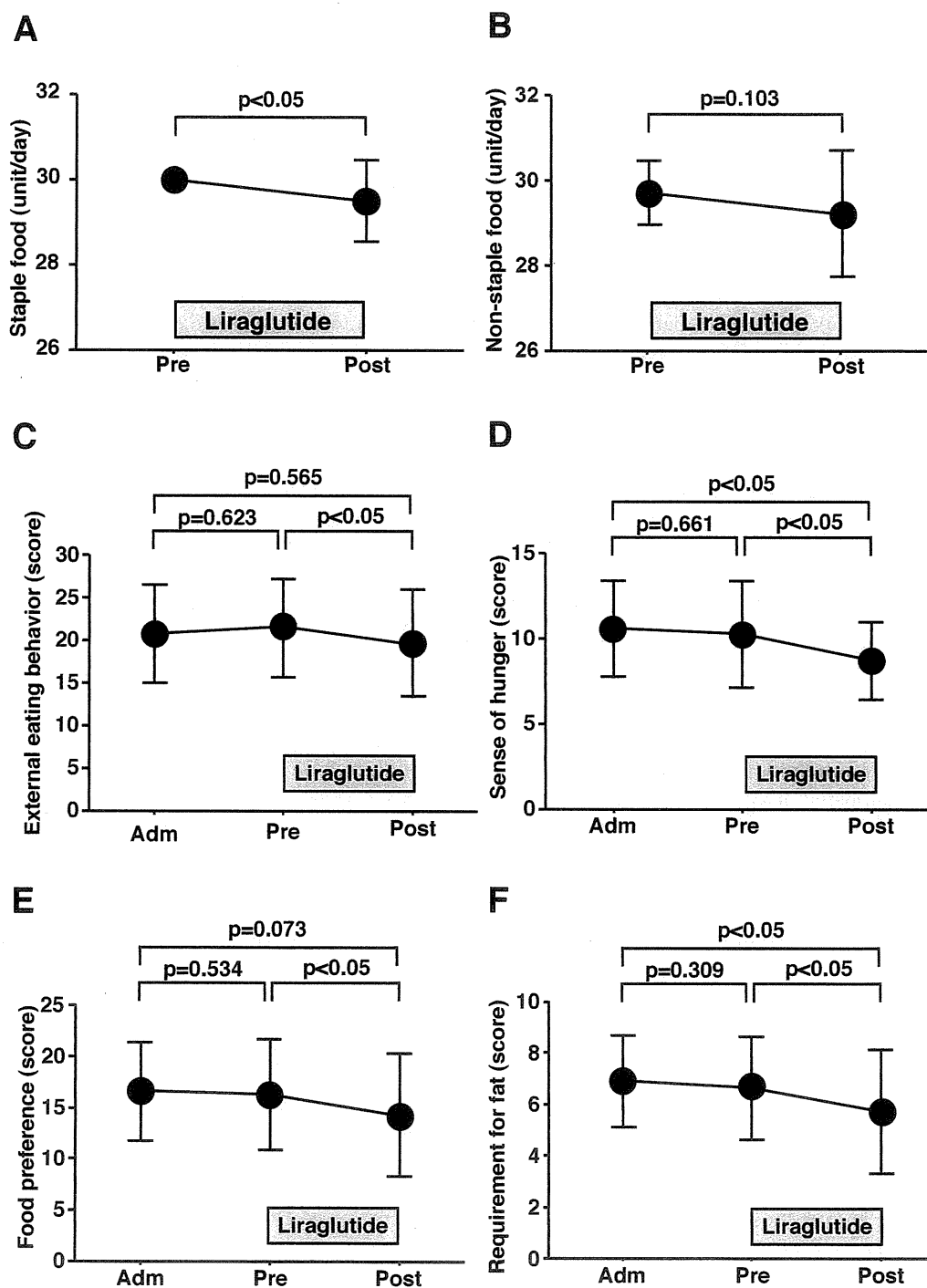


Figure 2 Liraglutide-induced changes in food intake and appetite. Every intake of staple food and non-staple food was rated on an eleven-point scale ranging from 0 (no intake) to 10 (full intake), respectively. Eating behavior was assessed by using a questionnaire as described in method section. Data are mean \pm 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/m² [22]. As shown in Table 1 the mean BMI of our patients was 28.3 kg/m², 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 liraglutide 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

on heart function and/or cardiomyocytes, although the GLP-1 action on the vascular system has not been fully evaluated especially in human. Treatment with GLP-1 improved endothelial dysfunction in type 2 diabetics with ischemic heart diseases [33]. Interestingly, GLP-1 treatment improved postprandial hyperlipidemia, suggesting the possibility that GLP-1 administration may reduce cardiovascular disease risk in type 2 diabetes [34]. A recent experiment showed that GLP-1 treatment protected against the development of atherosclerosis both *in vivo* and *in vitro* [35,36]. In the present study, liraglutide decreased serum hsCRP and sICAM-1 levels, while no such changes were observed following treatment with other glucose-lowering agents with insulin (n = 6), biguanide (BG) (n = 4), α -glucosidase inhibitor (α GI) (n = 3), dipeptidyl peptidase-4 inhibitors (DPP-4i) (n = 3), and sulfonylurea (SU) (n = 2) (data not shown). These results suggest that the decrease in hsCRP and sICAM-1 levels may be due to the pleiotropic effects of liraglutide, beneficial for protection against the development of atherosclerosis.

The present study has several limitations. The study is not a randomized clinical trial (RCT) and is not a crossover study. A crossover clinical trial will confirm present results. In addition, present study was performed in a small population. The effects of liraglutide on visceral fat adiposity, appetite, and cardiovascular biomarkers were examined in a hospitalized term. Collectively, a long-term RCT in obese Japanese type 2 diabetics should be conducted to confirm the efficacy of liraglutide on visceral fat adiposity, appetite, food behavior, and cardiovascular events.

In summary, short-term treatment with liraglutide effectively reduced visceral fat adiposity, appetite, and cardiovascular biomarkers in obese Japanese patients with type 2 diabetes. Longer term randomized clinical trials are warranted to more thoroughly elucidate the effect of liraglutide on these parameters.

Acknowledgements

We thank Miyuki Nakamura, Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, for the excellent technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research (C) no. 22590979 (to N. M.) and Scientific Research on Innovative Areas no. 22126008 (to T. F.). This article is dedicated to the memory of Dr. Hironobu Yoshimatsu, who was a professor of Oita University and proposed the questionnaire of eating behavior.

Author details

¹Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, 2-2-B5, Yamada-oka, Suita, Osaka 565-0871, Japan. ²Department of

Metabolism and Atherosclerosis, Graduate School of Medicine, Osaka University, 2-2-B5, Yamada-oka, Suita, Osaka 565-0871, Japan.

Authors' contributions

KI acquired and analyzed data, and wrote the manuscript. NM conceived study, analyzed data, and wrote the manuscript. SK, YF, JK, AHS, KO, and AI acquired and researched data. TF and IS reviewed manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 13 October 2011 Accepted: 1 December 2011

Published: 1 December 2011

References

1. Hossain P, Kowar B, El Nahas M: Obesity and diabetes in the developing world—a growing challenge. *N Engl J Med* 2007, **356**:213-215.
2. Chan JC, Malik V, Jia W, Kadowaki T, Yajnik CS, Yoon KH, Hu FB: Diabetes in Asia: epidemiology, risk factors, and pathophysiology. *JAMA* 2009, **301**:2129-2140.
3. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I: Adiponectin and metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2004, **24**:29-33.
4. Chilton R, Wyatt J, Nandish S, Oliveros R, Lujan M: Cardiovascular comorbidities of type 2 diabetes mellitus: the potential of glucagon-like peptide-1-based therapies. *Am J Med* 2011, **124**:S35-S53.
5. Mensink RP, Zock PL, Kester AD, Katan MB: Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am J Clin Nutr* 2003, **77**:1146-55.
6. Tanasescu M, Cho E, Manson JE, Hu FB: Dietary fat and cholesterol and the risk of cardiovascular disease among women with type 2 diabetes. *Am J Clin Nutr* 2004, **79**:999-1005.
7. Thanopoulou AC, Karamanos BG, Angelico FV, Assaad-Khalil SH, Barbato AF, Del Ben MP, Djordjevic PB, Dimitrijevic-Sreckovic VS, Gallotti CA, Katsilambros NL, Migdalis IN, Mrabet MM, Petkova MK, Roussi DP, Tenconi MT: Dietary fat intake as risk factor for the development of diabetes: multinational, multicenter study of the Mediterranean Group for the Study of Diabetes (MGSD). *Diabetes Care* 2003, **26**:302-7.
8. Meyer KA, Kushi LH, Jacobs DR Jr, Folsom AR: Dietary fat and incidence of type 2 diabetes in older Iowa women. *Diabetes Care* 2001, **24**:1528-35.
9. Devore EE, Stampfer MJ, Breteler MM, Rosner B, Hee Kang J, Okereke O, Hu FB, Grodstein F: Dietary fat intake and cognitive decline in women with type 2 diabetes. *Diabetes Care* 2009, **32**:635-40.
10. Agersø H, Jensen LB, Elbrønd B, Rolan P, Zdravkovic M: The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. *Diabetologia* 2002, **45**:195-202.
11. Holst JJ, Orskov C: The incretin approach for diabetes treatment: modulation of islet hormone release by GLP-1 agonism. *Diabetes* 2004, **53**:S197-S204.
12. Abu-Hamdan R, Rabiee A, Meneilly GS, Shannon RP, Andersen DK, Elahi D: Clinical review: The extrapancreatic effects of glucagon-like peptide-1 and related peptides. *J Clin Endocrinol Metab* 2009, **94**:1843-1852.
13. Kanoski SE, Fortin SM, Arnold M, Grill HJ, Hayes MR: Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. *Endocrinology* 2011, **152**:3103-12.
14. Ryo M, Maeda K, Onda T, Katashima M, Okumiya A, Nishida M, Yamaguchi T, Funahashi T, Matsuzawa Y, Nakamura T, Shimomura I: A new simple method for the measurement of visceral fat accumulation by bioelectrical impedance. *Diabetes Care* 2005, **28**:451-453.
15. Matsuda M, DeFronzo RA: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999, **22**:1462-1470.
16. Bergman RN, Ader M, Huecking K, Van Citters G: Accurate assessment of beta-cell function: the hyperbolic correction. *Diabetes* 2002, **51**:S212-S220.
17. Nauck M, Frid A, Hermansen K, Shah NS, Tankova T, Mitha IH, Zdravkovic M, Düring M, Matthews DR, Lead-2 Study Group: Efficacy and safety comparison of liraglutide, glimepiride, and placebo, all in combination

- with metformin, in type 2 diabetes: the LEAD (liraglutide effect and action in diabetes)-2 study. *Diabetes Care* 2009, **32**:84-90.
18. Jendle J, Nauck MA, Matthews DR, Frid A, Hermansen K, Düring M, Zdravkovic M, Strauss BJ, Garber AJ, LEAD-2 and LEAD-3 Study Groups: Weight loss with liraglutide, a once-daily human glucagon-like peptide-1 analogue for type 2 diabetes treatment as monotherapy or added to metformin, is primarily as a result of a reduction in fat tissue. *Diabetes Obes Metab* 2009, **11**:1163-72.
 19. Hansotia T, Maida A, Flock G, Yamada Y, Tsukiyama K, Seino Y, Drucker DJ: Extrapancratic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. *J Clin Invest* 2007, **117**:143-152.
 20. Kim Chung Ie T, Hosaka T, Yoshida M, Harada N, Sakae H, Sakai T, Nakaya Y: Exendin-4, a GLP-1 receptor agonist, directly induces adiponectin expression through protein kinase A pathway and prevents inflammatory adipokine expression. *Biochem Biophys Res Commun* 2009, **390**:613-618.
 21. Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, Wintle ME, Maggs DG: Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. *Curr Med Res Opin* 2008, **24**:275-286.
 22. Seino Y, Rasmussen MF, Zdravkovic M, Kaku K: Dose-dependent improvement in glycemia with once-daily liraglutide without hypoglycemia or weight gain: A double-blind, randomized, controlled trial in Japanese patients with type 2 diabetes. *Diabetes Res Clin Pract* 2008, **81**:161-168.
 23. Raun K, von Voss P, Gotfredsen CF, Golozoubova V, Rolin B, Knudsen LB: Liraglutide, a long-acting glucagon-like peptide-1 analog, reduces body weight and food intake in obese candy-fed rats, whereas a dipeptidyl peptidase-IV inhibitor, vildagliptin, does not. *Diabetes* 2007, **56**:8-15.
 24. Raun K, von Voss P, Knudsen LB: Liraglutide, a once-daily human glucagon-like peptide-1 analog, minimizes food intake in severely obese minipigs. *Obesity (Silver Spring)* 2007, **15**:1710-1716.
 25. Flint A, Raben A, Astrup A, Holst JJ: Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. *J Clin Invest* 1998, **101**:515-520.
 26. Toft-Nielsen MB, Madsbad S, Holst JJ: Continuous subcutaneous infusion of glucagon-like peptide 1 lowers plasma glucose and reduces appetite in type 2 diabetic patients. *Diabetes Care* 1999, **22**:1137-1143.
 27. Gutzwiller JP, Drewe J, Göke B, Schmidt H, Rohrer B, Lareida J, Beglinger C: Glucagon-like peptide-1 promotes satiety and reduces food intake in patients with diabetes mellitus type 2. *Am J Physiol* 1999, **276**:R1541-1544.
 28. Verdich C, Flint A, Gutzwiller JP, Näslund E, Beglinger C, Hellström PM, Long SJ, Morgan LM, Holst JJ, Astrup A: A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. *J Clin Endocrinol Metab* 2001, **86**:4382-4389.
 29. Kawate R, Yamakido M, Nishimoto Y, Bennett PH, Hamman RF, Knowler WC: Diabetes mellitus and its vascular complications in Japanese migrants on the Island of Hawaii. *Diabetes Care* 1979, **2**:161-70.
 30. Marshall JA, Hamman RF, Baxter J: High-fat, low-carbohydrate diet and the etiology of non-insulin-dependent diabetes mellitus: the San Luis Valley Diabetes Study. *Am J Epidemiol* 1991, **134**:590-603.
 31. Tsunehara CH, Leonetti DL, Fujimoto WY: Diet of second-generation Japanese-American men with and without non-insulin-dependent diabetes. *Am J Clin Nutr* 1990, **52**:731-8.
 32. Tinker LF, Bonds DE, Margolis KL, Manson JE, Howard BV, Larson J, Perri MG, Beresford SA, Robinson JG, Rodriguez B, Safford MM, Wenger NK, Stevens VJ, Parker LM: Women's Health Initiative. Low-fat dietary pattern and risk of treated diabetes mellitus in postmenopausal women: the Women's Health Initiative randomized controlled dietary modification trial. *Arch Intern Med* 2008, **168**:1500-11.
 33. Nyström T, Gutniak MK, Zhang Q, Zhang F, Holst JJ, Ahrén B, Sjöholm A: Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease. *Am J Physiol Endocrinol Metab* 2004, **287**:E1209-E1215.
 34. Ansar S, Koska J, Reaven PD: Postprandial hyperlipidemia, endothelial dysfunction and cardiovascular risk: focus on incretins. *Cardiovasc Diabetol* 2011, **10**:61.
 35. Arakawa M, Mita T, Azuma K, Ebato C, Goto H, Nomiya T, Fujitani Y, Hirose T, Kawamori R, Watada H: Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. *Diabetes* 2010, **59**:1030-1037.
 36. Gaspari T, Liu H, Welungoda I, Hu Y, Widdop RE, Knudsen LB, Simpson RW, Dear AE: A GLP-1 receptor agonist liraglutide inhibits endothelial cell dysfunction and vascular adhesion molecule expression in an ApoE^{-/-} mouse model. *Diab Vasc Dis Res* 2011, **8**:117-124.

doi:10.1186/1475-2840-10-109

Cite this article as: Inoue *et al.*: Short-term effects of liraglutide on visceral fat adiposity, appetite, and food preference: a pilot study of obese Japanese patients with type 2 diabetes. *Cardiovascular Diabetology* 2011 **10**:109.

Submit your next manuscript to BioMed Central and take full advantage of:

- convenient online submission
- thorough peer review
- immediate publication on BioMed Central
- open access: articles freely available online
- high visibility within the field
- Retaining the copyright to your article

Submit your manuscript at
www.biomedcentral.com/submit



Health Education “Hokenshido” Program Reduced Metabolic Syndrome in the Amagasaki Visceral Fat Study. Three-Year Follow-up Study of 3,174 Japanese Employees

Miwa Ryo¹, Tadashi Nakamura¹, Tohru Funahashi¹, Midori Noguchi², Ken Kishida¹, Yukiyoishi Okauchi¹, Hitoshi Nishizawa¹, Tomoko Ogawa², Sumi Kojima², Tetsuya Ohira³, Kohei Okita¹, Hiromi Iwahashi¹, Akihisa Imagawa¹, Yuji Matsuzawa⁴ and Ichiro Shimomura¹

Abstract

Objective The aim of this study was to evaluate the effects of health checkup and the health education “Hokenshido” program based on the concept that visceral fat accumulation causes metabolic syndrome (MetS), leading to cardiovascular disease (CVD).

Methods and Subjects Based on the Japanese definition of metabolic syndrome, in the annual health checkup for general subjects, the measurement of waist circumference and use of “Where am I?” chart on the way to develop atherosclerosis were introduced. The study group comprised 3,174 Japanese employees [2,440 males (46±11 years, mean ± SD), 734 females (43±10 years)], who underwent annual health checkup in 2003, 2004, and 2005. The medical staff provided “Hokenshido” for subjects assessed as having MetS and/or at high risk for CVD.

Results The prevalence of the MetS in 2003, 2004 and 2005 decreased in males (20.8%, 17.2%, 14.4%, $p < 0.001$) and females (3.0%, 2.2%, 1.9%, $p = 0.359$), respectively. Among subjects with MetS at baseline, the number of subjects with MetS significantly decreased in males (508, 287, 247, $p < 0.0001$) and females (22, 8, 6, $p < 0.0001$), respectively. Mean waist loss was 1.6 cm in males (< 0.0001) and 1.5 cm in females (< 0.001). Among subjects with metabolic syndrome at baseline, the mean waist loss was 2.5 cm in males (< 0.0001) and 3.9 cm in females (< 0.05). Fatal atherosclerotic vascular events were not recorded in this study period.

Conclusion Health check-up and the “Hokenshido” program reduced the prevalence of the MetS, which might lead to prevention of CVD.

Key words: metabolic syndrome, health checkup, health guidance, Hokenshido, visceral fat accumulation, cardiovascular disease

(Intern Med 50: 1643-1648, 2011)

(DOI: 10.2169/internalmedicine.50.5039)

Introduction

The metabolic syndrome is a risk factor for atherosclerotic cardiovascular diseases (CVD) (1). Visceral fat accumulation caused by overnutrition and physical inactivity is

closely related to glucose intolerance, dyslipidemia, hypertension, and CVD (2-4). Once a diagnosis of the metabolic syndrome is established, management of the condition, lifestyle change as the primary intervention and assessment of cardiovascular risk factors should be adequately conducted to reduce the risk of CVD (5-7).

¹Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Japan, ²Amagasaki City Office, Environment & Civic Affairs Bureau Health Support Promotion Section, Japan, ³Department of Social and Environmental Medicine, Graduate School of Medicine, Osaka University, Japan and ⁴Sumitomo Hospital, Japan

Received for publication December 21, 2010; Accepted for publication April 4, 2011

Correspondence to Dr. Miwa Ryo, ryomw@gaia.eonet.ne.jp