

correlated with homeostasis model assessment β -cell function (HOMA- β). Because SUIIT resembles HOMA- β in that insulin secretion is assumed to be suppressed to zero at approximately 60 mg/dL glucose in the formula, CPI might be a better index of residual β -cell mass than SUIIT in subjects with glucose intolerance. Furthermore, CPI is not affected by exogenous insulin²⁷, which might favor reproducibility of the results in patients with insulin therapy. Determination of the index using a one-point blood sample without the use of loading agents also favors CPI.

In results derived from CPI of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge, AUC was 0.75, optimal cut-off value was 1.1 ng/mg with 61% sensitivity and 78% specificity, and values at 90% sensitivity and at 90% specificity were 1.7 and 0.7 ng/mg, respectively. Interestingly, in results derived from CPI of patients who achieved <7.4% HbA_{1c} within 6 months after discharge, AUC was 0.75, optimal cut-off value was 1.2 ng/mg with 73% sensitivity and 71% specificity, and values at 90% sensitivity and at 90% specificity were 1.7 and 0.8 ng/mg, respectively, similar to the values evaluated by mean preprandial glucose levels at discharge. These values are also similar to those in a previous report in Japanese using the data of 180 subjects from another institution (optimal cut-off value: 1.0 with 62% sensitivity and 81% specificity; values at 90% sensitivity: 1.8; 90% specificity: 0.7 ng/mg), although good glycemic control was defined as 8.4% in HbA_{1c}, which is somewhat inadequate¹⁴. Thus, CPI might be a predictor of suitable therapy to achieve fair glycemic control not only for the short-term, but also for longer duration.

The main limitation of the present study is that it is a retrospective analysis of inpatients at one hospital, and the protocol for starting insulin therapy was not defined precisely. However, in the achieved group analyzed as subjects, the decisions as to whether to start insulin therapy made by Japanese Board Certified Diabetologists were confirmed retrospectively to have been made according to the treatment guide for diabetes of the Japan Diabetes Society, as discussed in the results section.

In conclusion, we have shown the advantage of CPI of indices using CPR to select insulin therapy to achieve good glycemic control. However, limitations of the predictive abilities of indices using CPR generally and the importance of observation of the clinical therapeutic course must be taken into consideration.

ACKNOWLEDGEMENTS

The authors declare no conflict of interest.

REFERENCES

- DeFronzo RA. Lilly lecture 1987. The triumvirate: β -cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 1988; 37: 667–687.
- Yki-Järvinen H, Kauppila M, Kujansuu E, *et al.* Comparison of insulin regimens in patients with non-insulin-dependent diabetes mellitus. *N Engl J Med* 1992; 327: 1426–1433.
- Turner RC, Cull CA, Frighi V, *et al.* Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. *JAMA* 1999; 281: 2005–2012.
- Japan Diabetes Society. Diabetes mellitus: the disease itself. In: Japan Diabetes Society (ed.). *Treatment Guide for Diabetes 2007*. Bunkodo, Japan, 2007; 8–13.
- U.K. Prospective Diabetes Study Group. U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. *Diabetes* 1995; 44: 1249–1258.
- Funakoshi S, Fujimoto S, Hamasaki A, *et al.* Analysis of factors influencing pancreatic β -cell function in Japanese patients with type 2 diabetes: association with body mass index and duration of diabetic exposure. *Diabetes Res Clin Pract* 2008; 82: 353–358.
- Faber OK, Binder C. C-peptide response to glucagon. A test for the residual β -cell function in diabetes mellitus. *Diabetes* 1977; 26: 605–610.
- Hendriksen C, Faber OK, Drejer J, *et al.* Prevalence of residual B-cell function in insulin-treated diabetics evaluated by the plasma C-peptide response to intravenous glucagon. *Diabetologia* 1977; 13: 615–619.
- Jayyab AK, Heding LG, Czyzyk A, *et al.* Serum C peptide and IRI levels after administration of glucagon and glucose in non-insulin-dependent diabetics. *Horm Metab Res* 1982; 14: 112–116.
- Gjessing HJ, Damsgaard EM, Matzen LE, *et al.* Reproducibility of β -cell function estimates in non-insulin-dependent diabetes mellitus. *Diabetes Care* 1987; 10: 558–562.
- Madsbad S, Krarup T, McNair P, *et al.* Practical clinical value of the C-peptide response to glucagon stimulation in the choice of treatment in diabetes mellitus. *Acta Med Scand* 1981; 210: 153–156.
- Sanke T, Satogami E, Sowa R, *et al.* Plasma C-peptide response during glucagon test as an index for evaluation of insulin requirement in diabetics (in Japanese). *J Jpn Diab Soc* 1985; 28: 713–719.
- Koskinen P, Viikari J, Irjala K, *et al.* C-peptide determination in the choice of treatment in diabetes mellitus. *Scand J Clin Lab Invest* 1985; 45: 589–597.
- Asano T, Kawamura M, Watanabe T, *et al.* Indices of urinary and serum C-peptide corrected with fasting plasma glucose for decision-making of insulin therapy in type 2 diabetes-validation and comparison (in Japanese). *J Jpn Diab Soc* 2008; 51: 759–763.
- American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33: S62–S69.
- Kajinuma H, Tanabashi S, Ishiwata K, *et al.* Urinary excretion of C-peptide in relation to renal function. In: Baba S (ed.). *Proinsulin, Insulin, C-Peptide*. Excerpta Medica, Amsterdam, 1979; 183–189.
- American Diabetes Association. Standards of medical care in diabetes-2010. *Diabetes Care* 2010; 33: S11–S61.
- Japan Diabetes Society (ed.). *Treatment Guide for Diabetes 2007*. Bunkodo, Japan, 2007.

19. The Committee of Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Invest* 2010; 1: 212–228.
20. Yamada Y, Fukuda K, Fujimoto S, *et al.* SUIT, secretory units of islets in transplantation: an index for therapeutic management of islet transplanted patients and its application to type 2 diabetes. *Diabetes Res Clin Pract* 2006; 74: 222–226.
21. Japan Diabetes Society. Insulin treatment. In: Japan Diabetes Society (ed.). *Treatment Guide for Diabetes 2007*. Bunkodo, Japan, 2007; 47–55.
22. Franz MJ, Monk A, Barry B, *et al.* Effectiveness of medical nutrition therapy provided by dietitians in the management of non-insulin-dependent diabetes mellitus: a randomized, controlled clinical trial. *J Am Diet Assoc* 1995; 95: 1009–1017.
23. Ziemer DC, Berkowitz KJ, Panayiotou RM, *et al.* A simple meal plan emphasizing healthy food choices is as effective as an exchange-based meal plan for urban African Americans with type 2 diabetes. *Diabetes Care* 2003; 26: 1719–1724.
24. Miller CK, Edwards L, Kissling G, *et al.* Nutrition education improves metabolic outcomes among older adults with diabetes mellitus: results from a randomized controlled trial. *Prev Med* 2002; 34: 252–259.
25. Japan Diabetes Society. Diet therapy. In: Japan Diabetes Society (ed.). *Treatment Guide for Diabetes 2007*. Bunkodo, Japan, 2007; 34–37.
26. Park SW, Ihm SH, Yoo HJ, *et al.* Differential effects of ambient blood glucose level and degree of obesity on basal serum C-peptide level and the C-peptide response to glucose and glucagon in non-insulin-dependent diabetes mellitus. *Diabetes Res Clin Pract* 1997; 37: 165–171.
27. Albareda M, Rigla M, Rodríguez-Espinosa J, *et al.* Influence of exogenous insulin on C-peptide levels in subjects with type 2 diabetes. *Diabetes Res Clin Pract* 2005; 68: 202–206.
28. Peacock I, Tattersall RB. The difficult choice of treatment for poorly controlled maturity onset diabetes: tablets or insulin? *Br Med J (Clin Res Ed)* 1984; 288: 1956–1959.
29. Sakuraba H, Mizukami H, Yagihashi N, *et al.* Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 2002; 45: 85–96.
30. Butler AE, Janson J, Bonner-Weir S, *et al.* β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003; 52: 102–110.
31. Rahier J, Guiot Y, Goebbels RM, *et al.* Pancreatic β -cell mass in European subjects with type 2 diabetes. *Diabetes Obes Metab* 2008; 10: 32–42.
32. Meier JJ, Menge BA, Breuer TG, *et al.* Functional assessment of pancreatic β -cell area in humans. *Diabetes* 2009; 58: 1595–1603.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | Process of selection of subjects for analysis.

Figure S2 | Therapeutic modes of analyzed patients at admission and discharge, and the required alteration of therapy during the period of admission.

Figure S3 | Relative frequency distribution of C-peptide indices of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge in the non-insulin and insulin group.

Table S1 | Details of medication and daily dosages of oral hypoglycemic agents used at discharge

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Plasma gastric inhibitory polypeptide and glucagon-like peptide-1 levels after glucose loading are associated with different factors in Japanese subjects

Norio Harada, Akihiro Hamasaki, Shunsuke Yamane, Atsushi Muraoka, Erina Joo, Kazuyo Fujita, Nobuya Inagaki*

ABSTRACT

Aims/Introduction: Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are major incretins that potentiate insulin secretion from pancreatic β -cells. The factors responsible for incretin secretion have been reported in Caucasian subjects, but have not been thoroughly evaluated in Japanese subjects. We evaluated the factors associated with incretin secretion during oral glucose tolerance test (OGTT) in Japanese subjects with normal glucose tolerance (NGT).

Materials and Methods: We measured plasma GIP and GLP-1 levels during OGTT in 17 Japanese NGT subjects and evaluated the factors associated with GIP and GLP-1 secretion using simple and multiple regression analyses.

Results: GIP secretion (AUC-GIP) was positively associated with body mass index ($P < 0.05$), and area under the curve (AUC) of C-peptide ($P < 0.05$) and glucagon ($P < 0.01$), whereas GLP-1 secretion (AUC-GLP-1) was negatively associated with AUC of plasma glucose ($P < 0.05$). The insulinogenic index was most strongly associated with GIP secretion ($P < 0.05$); homeostasis model assessment β -cell was the most the strongly associated factor in GLP-1 secretion ($P < 0.05$) among the four indices of insulin secretion and insulin sensitivity.

Conclusions: Several distinct factors might be associated with GIP and GLP-1 secretion during OGTT in Japanese subjects. (*J Diabetes Invest*, doi: 10.1111/j.2040-1124.2010.00078.x, 2010)

KEY WORDS: Gastric inhibitory polypeptide, Glucagon-like peptide-1, Incretin

INTRODUCTION

Oral glucose administration leads to greater insulin release from pancreatic islets than intravenous glucose loading that yields equivalent glucose levels. Gut hormonal substances released in response to glucose include the incretins, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are responsible for 50–60% of postprandial insulin secretion¹. GIP is secreted on meal ingestion from K-cells in the proximal small intestine, whereas GLP-1 is secreted from L-cells in the distal small intestine and colon, and binds to their respective receptors (GIP receptor [GIPR] and GLP-1 receptor) on the surface of pancreatic β -cells to stimulate insulin secretion by increasing the intracellular adenosine 3',5'-monophosphate (cAMP) concentration^{2–4}.

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity^{5–7}. The incretin effect has been shown to be reduced in type 2 diabetic subjects com-

pared with those with normal glucose tolerance (NGT) in previous studies^{8,9}, suggesting that a reduced incretin effect might be associated with hyperglycemia after food intake and glucose loading in type 2 diabetes. When intravenous infusion of GIP or GLP-1 was carried out in type 2 diabetic subjects, GLP-1 potentiated insulin secretion from pancreatic β -cells, but GIP did not, showing that the GIPR signal is downregulated in β -cells in type 2 diabetes¹⁰. In studies using rodent models, it was reported that GIPR mRNA and protein expression levels in islets are decreased in the diabetic state¹¹. In contrast, in the non-diabetic obese state, GIP plays an important role in maintaining blood glucose levels¹². The GIP signal might be enhanced as a result of increased GIPR sensitivity of β -cells to GIP or increased GIP secretion from K-cells in the non-diabetic obese state. Indeed, GIP concentrations are reported to be increased in obese rodent models and human subjects compared with those in lean rodents and human subjects, respectively^{13–15}. Furthermore, we have previously shown the hypersensitivity of GIPR to GIP in β -cells of high fat-induced obese mice¹⁶. Plasma GLP-1 concentrations in type 2 diabetic patients are reported to be reduced after meal ingestion and glucose loading^{9,17}. However, in other studies it was reported that GLP-1 concentrations did not differ

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

*Corresponding author. Nobuya Inagaki Tel.: +81-75-751-3560 Fax: +81-75-751-4244 E-mail address: inagaki@metab.kuhp.kyoto-u.ac.jp

Received 12 August 2010; revised 16 September 2010; accepted 16 September 2010

in NGT and type 2 diabetic subjects^{18–20}. Thus, the measurement of GIP and GLP-1 concentrations in various metabolic states is important to evaluate the effects of incretin on insulin secretion.

Insulin sensitivity in Asian subjects has been shown to be higher than in Mexican Americans and Caucasians in previous reports^{21,22}, which is partly as a result of the fact that Asians, including Japanese, are generally less obese. Furthermore, insulin secretion rather than insulin sensitivity is the more important factor in progression from NGT to diabetes in Japanese subjects²³. We have reported that early-phase insulin secretion is considerably decreased, even in Japanese NGT subjects with 1-h plasma glucose (PG) levels during oral glucose tolerance test (OGTT) of more than 180 mg/dL²⁴. Thus, it is especially important to evaluate incretin secretion and determine the factors associated with incretin secretion in Japanese NGT subjects, because GIP and incretin is responsible for more than 50% of postprandial insulin secretion after glucose ingestion. The factors responsible for incretin secretion have been reported in Caucasian subjects, but have not been thoroughly elucidated in Japanese subjects.

In the present study, we evaluated GIP and GLP-1 levels during OGTT and determined the factors involved in GIP and GLP-1 secretion (area under the curve [AUC] of GIP and GLP-1 during OGTT) in Japanese NGT subjects.

MATERIALS AND METHODS

Subjects

We recruited 17 Japanese healthy volunteers. The subjects had no history of hypertension, hyperlipidemia or kidney and liver diseases, and did not take any drugs 2 weeks before the study. The study was designed in compliance with the ethics regulations of the Helsinki Declaration and Kyoto University. Informed consent was obtained from all subjects.

Study Procedure

The subjects' age, height and bodyweight were determined. Blood samples for the measurement of liver and kidney function, HbA_{1c}, serum triglyceride (TG), total cholesterol and high-density lipoprotein (HDL)-cholesterol levels were drawn after an overnight fast. All subjects received OGTT. After the subjects fasted overnight for 10–16 h, standard OGTT with 75 g glucose was given according to the National Diabetes Data Group recommendations²⁵. NGT was diagnosed according to World Health Organization (WHO) criteria²⁶.

Blood samples were collected at –15, 0, 10, 20, 30, 60, 90, 120, 150 and 180 min after glucose loading and were centrifuged at 1800 g at 4°C for 10 min. After collecting supernatant of the samples, plasma and serum were stocked at –80°C. Plasma GIP, GLP-1 levels and the various parameters (PG, serum immunoreactive insulin [IRI], serum C-peptide reactivity [CPR], TG, serum free fatty acid [FFA] and plasma glucagon) were measured at the indicated times (plasma GIP and GLP-1 levels were measured at –15, 0, 10, 30, 60, 90, 120 and 180 min after glucose loading, and plasma glucagon levels were measured

at –15, 0, 30, 60, 90, 120 and 180 min after glucose loading). The PG levels were measured by glucose oxidase method. Serum IRI levels were measured by two-site radioimmunoassay. Total GIP and total GLP-1 levels were measured using human GIP ELISA kit (Linco Research, St Charles, MO, USA; range of detection from 8.2 pg/mL to 2000 pg/dL) and human GLP-1 ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA; range of detection from 2.4 pg/mL to 1,000,000 pg/dL), respectively, as previously described^{27,28}. The AUC of PG, IRI, CPR, TG, FFA, glucagon, total GIP (AUC-GIP) and total GLP-1 (AUC-GLP-1) were calculated. We then analyzed the relationship between the AUC of GIP (GIP secretion) and GLP-1 (GLP-1 secretion) and age, body mass index (BMI) and the parameters during OGTT.

Statistical Analysis

Basal insulin secretion and sensitivity were evaluated by homeostasis model assessment (HOMA) β -cell function and homeostasis model assessment of insulin resistance (HOMA-IR)^{29,30}, respectively. Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index³¹ and insulin sensitivity index (ISI) composite³². The calculations of the four indices were as follows:

$$\text{HOMA } \beta\text{-cell} = 20 \times \text{fasting IRI level (FIRI) (pmol/L)} / (\text{fasting PG level [FPG] [mmol/L]} - 3.5)$$

$$\text{HOMA-IR} = \text{FIRI (pmol/L)} \times \text{FPG (mmol/L)} / 22.5$$

$$\text{Insulinogenic index} = (30 \text{ min IRI} - \text{FIRI [pmol/L]}) / (30 \text{ min PG} - \text{FPG [mmol/L]})$$

$$\begin{aligned} \text{IRI composite} &= 10,000 / (\text{FPG [mg/dL]} \times \text{FIRI [\mu U/mL]} \\ &\quad \times \text{mean OGTT PG [mg/dL]} \\ &\quad \times \text{mean OGTT IRI [\mu U/mL]})^{0.5} \end{aligned}$$

All analyses were carried out using statistical analysis software (SPSS version 17.0, IBM, Somers, NY, USA) system. Statistical analysis was carried out by ANOVA with Fisher's PLSD test for changing levels of GIP, GLP-1, and the parameters during OGTT and differences between the two groups were assessed by unpaired *t*-test. We used simple regression analysis to determine the relationship between AUC-GIP or AUC-GLP-1 and the age, BMI and the parameters during OGTT, and we carried out multiple regression analysis to determine the factors most strongly associated with AUC-GIP and AUC-GLP-1, and the indices of insulin secretion and sensitivity. Probability (*P*) values <0.05 were considered statistically significant. Data are presented as mean $\pm$ standard error (SE).

RESULTS

Table 1 shows clinical characteristics of the subjects. Mean age was 31.7 ± 1.3 years and mean BMI was 23.1 ± 0.9 kg/m². No subjects had liver or kidney dysfunction. HbA_{1c}, FPG, TG, total

Table 1 | Clinical characteristics of the subjects

<i>n</i> (male/female)	17 (14/3)
Age (years)	31.7 ± 1.3
Body mass index (kg/m ²)	23.1 ± 0.9
Fasting plasma glucose (mmol/L)	6.1 ± 0.2
Fasting insulin (pmol/L)	25.2 ± 3.7
HbA _{1c} (%)	4.7 ± 0.0
Triglycerides (mmol/L)	2.00 ± 0.31
Total cholesterol (mmol/L)	4.56 ± 0.16
HDL-cholesterol (mmol/L)	1.51 ± 0.10
Insulinogenic index	66.22 ± 8.54
HOMA β-cell	60.85 ± 8.89
HOMA-IR	0.94 ± 0.15
ISI composite	11.45 ± 1.67

Means ± SE. HDL, high-density lipoprotein; HOMA, homeostasis model assessment; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index.

cholesterol and HDL-cholesterol levels were within normal limits in the fasting state.

The levels of GIP, GLP-1, PG, IRI, CPR, TG, FFA and glucagon after glucose loading were measured (Figure 1). The subjects were diagnosed NGT according to WHO criteria with fasting plasma glucose and 2-h glucose levels below 6.1 and 7.8 mmol/L, respectively. Levels of PG, IRI and CPR were significantly increased from 10 min after glucose loading compared with fasting level (Figure 1a–c). FFA levels were significantly decreased from 10 min after glucose loading (Figure 1d). TG levels were not significantly changed during OGTT (Figure 1e). Glucagon levels were significantly decreased from 30 min after glucose loading (Figure 1f). Total GIP levels were significantly increased from 10 min during OGTT (Figure 1g). Total GLP-1

levels were significantly increased from 10 min during OGTT with peaks at 30 and 120 min (Figure 1h).

We analyzed the relationship between AUC-GIP or AUC-GLP-1 and age, BMI and the several parameters (AUC of PG, IRI, CPR, TG, FFA and glucagon). AUC-GIP were positively related to BMI and AUC of CPR, IRI and glucagon, but AUC-GLP-1 was not related to these factors (Figure 2a–c; AUC data of IRI during OGTT are not shown; $P < 0.05$). In contrast, AUC-GLP-1 was inversely related to AUC of PG (Figure 2d), but AUC-GIP was not.

We then analyzed the relationship between AUC-GIP or AUC-GLP-1 and indices of insulin secretion and insulin sensitivity. AUC-GIP was positively related to insulinogenic index and HOMA-IR, whereas AUC-GLP-1 was positively related to HOMA β-cell function (Figure 3a–c). ISI composite was not related to either AUC-GIP or AUC-GLP-1 (Figure 3d). In addition, multiple regression analysis was carried out to determine the factors strongly associated with AUC-GIP and AUC-GLP-1. The insulinogenic index was the most strongly associated factor in AUC-GIP (correlation coefficients 0.56, standardized β 0.56, $P < 0.05$) of the four indices; HOMA β-cell function was the strongest factor in AUC-GLP-1 (HOMA β-cell function: correlation coefficients 0.524, standardized β 0.870, $P < 0.01$, ISI composite: correlation coefficients 0.063, standardized β 0.581, $P < 0.05$).

DISCUSSION

In the present study, we estimated the incretin level after glucose loading in Japanese NGT subjects and found that plasma GIP and GLP-1 levels during OGTT are related to different factors.

Incretin action of GIP is reduced in the diabetic state as a result of decreased GIP receptor expression on pancreatic β-cells¹¹, whereas GIP signaling is enhanced and maintains

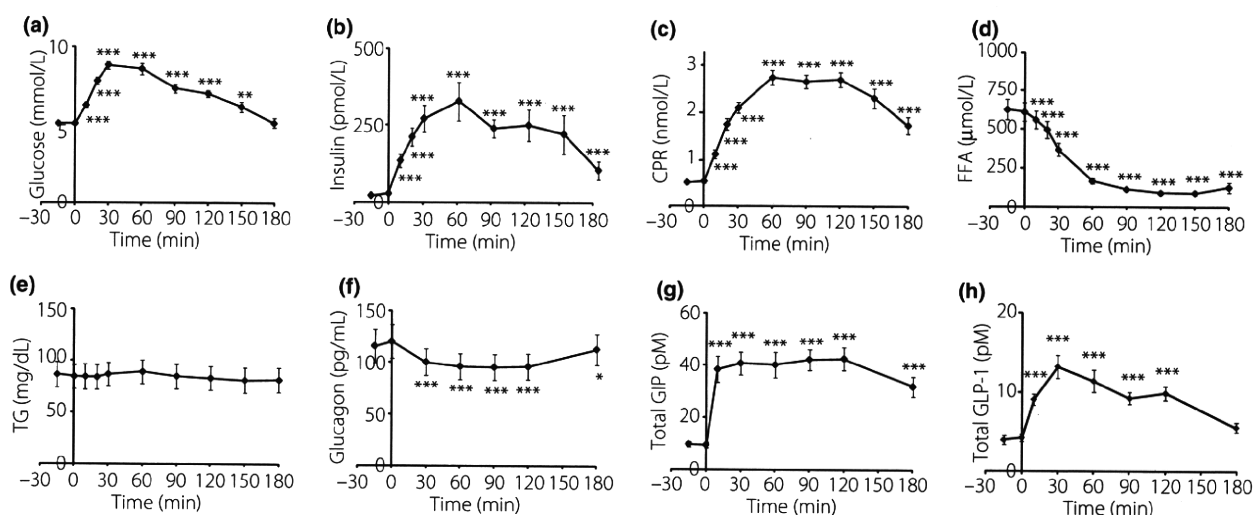


Figure 1 | Concentrations of (a) plasma glucose, (b) serum immunoreactive insulin, (c) serum C-peptide reactivity (CPR), (d) serum free fatty acid (FFA), (e) serum triglyceride (TG), (f) glucagon, (g) total gastric inhibitory polypeptide (GIP) and (h) total glucagon-like peptide-1 (GLP-1) during oral glucose tolerance test in 17 Japanese subjects. Mean ± SE, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the levels at fasting.

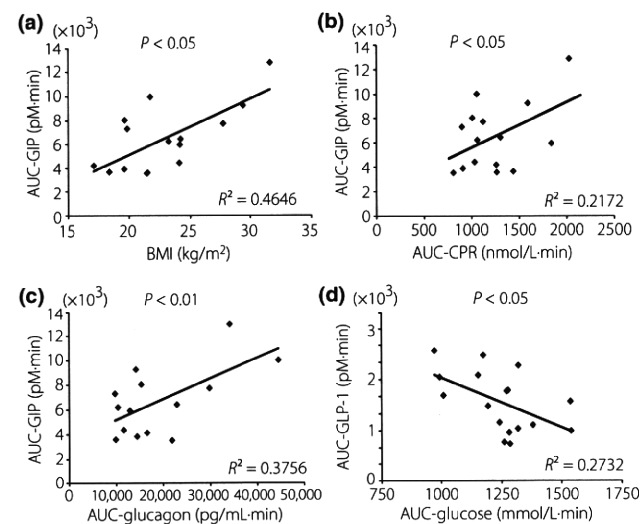


Figure 2 | Simple regression analysis of gastric inhibitory polypeptide secretion (AUC-GIP) and (a) body mass index (BMI), (b) AUC of serum C-peptide reactivity (CPR) and (c) glucagon. (d) Simple regression analysis of glucagon-like peptide-1 secretion (AUC-GLP-1) and AUC of plasma glucose (PG).

glucose homeostasis by compensatory increased insulin secretion in the obese state^{15,16}. In some human studies in Caucasians, plasma GIP levels are increased in obese subjects^{14,15} and there

is a positive relationship between AUC-GIP and AUC of FFA during OGTT¹⁸. In the present study, AUC-GIP after glucose loading was not associated with AUC of FFA, but was positively associated with BMI, HOMA-IR, and AUC of IRI and CPR after glucose loading. In fact, obese subjects are known to have hyperinsulinemia and insulin resistance^{33,34}, and BMI was strongly associated with AUC of IRI and CPR. Thus, GIP secretion from K-cells may well be associated with insulin resistance to maintain postprandial hyperinsulinemia in Japanese NGT subjects. It is unknown why there was no correlation between AUC-GIP and AUC-glucose. It might be explained by the fact that GIP secretion is associated with the amount of glucose loading¹, whereas blood glucose levels are maintained within normal levels by GIP-induced compensatory insulin secretion in NGT subjects.

GLP-1 secretions of type 2 diabetes subjects after glucose or meal ingestion are diverse in human studies^{9,17–19}. Some studies report that GLP-1 secretion is decreased in Caucasian type 2 diabetes^{9,17}. Recently, it is reported that GLP-1 levels after ingestion of glucose and mix meal in Japanese type 2 diabetic subjects were not decreased compared with those in NGT subjects, suggesting that GLP-1 secretion is not decreased in Japanese type 2 diabetes^{20,35,36}. Two studies of Caucasian subjects found that AUC-GLP-1 during OGTT is positively associated with age and AUC of glucagon, whereas AUC of GLP-1 is negatively associated with BMI or bodyweight and AUC of FFA^{9,18}. In the

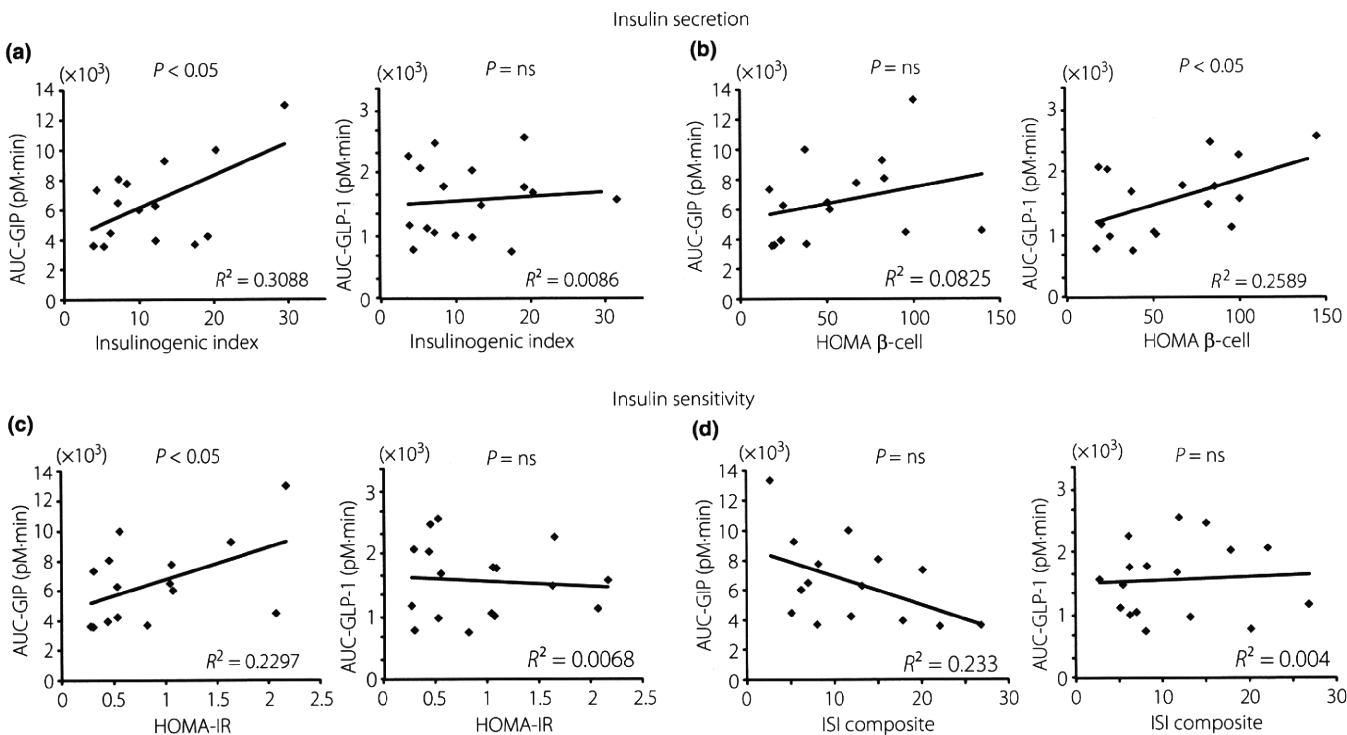


Figure 3 | Relationship between gastric inhibitory polypeptide secretion (AUC-GIP) and glucagon-like peptide-1 secretion (AUC-GLP-1) and the indices of insulin secretion and insulin sensitivity. (a) Insulinogenic index, (b) homeostasis model assessment (HOMA) β -cell function, (c) homeostasis model assessment of insulin resistance (HOMA-IR) and (d) insulin sensitivity index (ISI) composite. Ns, not significant.

present study, AUC-GLP-1 was negatively related to AUC of PG during OGTT, showing that the increase in GLP-1 secretion after glucose loading is associated with a decrease in postprandial glucose levels in Japanese NGT subjects. It has been reported that GLP-1 levels after glucose loading are positively related to gastric emptying in Caucasian subjects³⁷. Although we did not measure gastric emptying of the subjects in the present study, increasing GLP-1 secretion after glucose loading might decrease postprandial glucose levels through gastric emptying. In the present study, BMI and AUC of FFA were not associated with AUC-GLP-1 during OGTT. Obese subjects have higher FFA levels than lean subjects³⁸. However, because Japanese subjects are less obese than Caucasian subjects²¹, the difference observed in the relationship between AUC-GIP and GLP-1, and AUC of FFA might reflect this ethnic difference in Caucasians and Japanese.

Insulin secretion, rather than insulin sensitivity, is the more important factor in the progression from NGT to type 2 diabetes in Japanese patients^{23,39}. Because incretin is an intestinal hormone that induces postprandial insulin secretion¹, we hypothesize that GIP and GLP-1 secretion is more crucial in Japanese subjects than in Caucasian subjects. Indeed, GLP-1 mimetics and DPP-4 inhibitors improve glycemic control better in Japanese type 2 diabetic patients than in Caucasian type 2 diabetic patients in clinical trials^{40–43}. We therefore evaluated the correlation between GIP secretion (AUC-GIP) and GLP-1 secretion (AUC-GLP-1), and the indices of insulin secretion and insulin sensitivity in Japanese NGT subjects during OGTT. The values of HOMA β -cell, insulinogenic index, HOMA-IR and ISI composite were similar to those in previous studies of Japanese subjects^{24,30,39}. AUC-GIP was positively associated with the insulinogenic index and HOMA-IR, and the insulinogenic index was strongly associated with AUC-GIP, whereas AUC-GLP-1 was associated only with HOMA β -cell among the four indices. It has been reported that early-phase insulin secretion is an important factor in the progression from NGT through impaired glucose tolerance (IGT) to type 2 diabetes³⁹, and that basal insulin secretion (HOMA β -cell) and insulin resistance are important factors in the progression from NGT through impaired fasting glucose (IFG) to type 2 diabetes in Japanese patients⁴⁴. Thus, enhancing the GIP and GLP-1 signals might be particularly useful in inhibiting the progression of type 2 diabetes in Japanese patients. Recently, variants at the GIP receptor gene locus associated with 2-h glucose levels during OGTT were identified by meta-analysis of genome-wide association studies⁴⁵. In subjects who carry this GIP receptor risk allele, early-phase insulin secretion is decreased. These data seem to support our results that GIP secretion is associated with insulinogenic index in Japanese NGT subjects.

In conclusion, we evaluated plasma GIP and GLP-1 levels during OGTT in Japanese NGT subjects. GLP-1 secretion was associated with PG during OGTT, and basal insulin secretion (HOMA β -cell) and GIP secretion was associated with BMI and early-phase insulin secretion (insulinogenic index). Thus, there

might be different factors associated with GIP and GLP-1 secretion during OGTT in Japanese subjects.

ACKNOWLEDGEMENTS

We thank Dr Yutaka Seino (Kansai Electric Power Hospital) for his helpful suggestions. This study was supported by Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Kyoto University Global COE Program 'Center for Frontier Medicine', and also by Novo Nordisk Pharma Ltd.

REFERENCES

1. Nauck MA, Homberger E, Siegel EG, *et al.* Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab* 1986; 63: 492–498.
2. Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormone: similarities and difference. *J Diabetes Invest* 2010; 1: 8–23.
3. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2010; 3: 153–165.
4. Holst JJ. The physiology of glucagon-like peptide 1. *Physiol Rev* 2007; 87: 1409–1439.
5. Mittraku A, Kelley D, Mook M, *et al.* Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose tolerance. *N Engl J Med* 1992; 2: 22–29.
6. Haffner SM, Stern MP, Hazuda HP, *et al.* Increased insulin concentrations in nondiabetic offspring of diabetic parents. *N Engl J Med* 1988; 17: 1297–1301.
7. Saad MF, Knowler WC, Pettitt D, *et al.* A two-step model for development of non-insulin-dependent diabetes. *Am J Med* 1991; 90: 229–235.
8. Nauck M, Stöckmann F, Ebert R, *et al.* Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 1986; 29: 46–52.
9. Muscelli E, Mari A, Casolaro A, *et al.* Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 2008; 57: 1340–1348.
10. Nauck MA, Heimesaat MM, Orskov C, *et al.* Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 1993; 91: 301–307.
11. Holst JJ, Gromada J, Nauck MA. The pathogenesis of NIDDM involves a defective expression of the GIP receptor. *Diabetologia* 1997; 40: 984–986.
12. Miyawaki K, Yamada Y, Yano H, *et al.* Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci USA* 1999; 96: 14843–14847.
13. Miyawaki K, Yamada Y, Ban N, *et al.* Inhibition of gastric inhibitory polypeptide signaling prevents obesity. *Nat Med* 2002; 8: 738–742.

14. Flatt PR, Bailey CJ, Kwasowski P, et al. Abnormalities of GIP in spontaneous syndromes of obesity and diabetes in mice. *Diabetes* 1983; 32: 433–435.
15. Creutzfeldt W, Ebert R, Willms B, et al. Gastric inhibitory polypeptide (GIP) and insulin in obesity: increased response to stimulation and defective feedback control of serum levels. *Diabetologia* 1978; 14: 15–24.
16. Harada N, Yamada Y, Tsukiyama K, et al. A novel GIP receptor splice variant influences GIP sensitivity of pancreatic beta-cells in obese mice. *Am J Physiol Endocrinol Metab* 2008; 294: E61–E68.
17. Vilsbøll T, Krarup T, Deacon CF, et al. Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. *Diabetes* 2001; 50: 609–613.
18. Vollmer K, Holst JJ, Baller B, et al. Predictors of incretin concentrations in subjects with normal, impaired, and diabetic glucose tolerance. *Diabetes* 2008; 57: 678–687.
19. Faerch K, Vaag A, Holst JJ, et al. Impaired fasting glycaemia vs impaired glucose tolerance: similar impairment of pancreatic alpha and beta cell function but differential roles of incretin hormones and insulin action. *Diabetologia* 2008; 51: 853–861.
20. Yabe D, Kuroe A, Lee S, et al. Little enhancement of meal-induced glucagon-like peptide 1 secretion in Japanese: comparison of type 2 diabetes patients and healthy controls. *J Diabetes Invest* 2010; 1: 56–59.
21. Chiu KC, Chuang LM, Yoon C. Comparison of measured and estimated indices of insulin sensitivity and beta cell function: impact of ethnicity on insulin sensitivity and beta cell function in glucose-tolerant and normotensive subjects. *J Clin Endocrinol Metab* 2001; 86: 1620–1625.
22. Mandavilli A, Cyranoski D. Asian's big problem. *Nature Med* 2004; 10: 325–327.
23. Seino Y, Ikeda M, Yawata M, et al. The insulinogenic index in secondary diabetes. *Horm Metab Res* 1975; 7: 323–335.
24. Harada N, Fukushima M, Toyoda K, et al. Factors responsible for elevation of one hour postchallenge plasma glucose levels in Japanese men. *Diabetes Res Clin Pract* 2008; 81: 284–289.
25. National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 1979; 28: 1039–1057.
26. Alberi KG, Zimmerer PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 54: 539–553.
27. Narita T, Katsuura Y, Sato T, et al. Miglitol induces prolonged and enhanced glucagon-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.
28. Lim GE, Huang GJ, Flora N, et al. Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell. *Endocrinology* 2009; 150: 580–591.
29. Matthews DR, Hosker JP, Rudenski AS. Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412–419.
30. Fukushima M, Taniguchi A, Sakai M, et al. Homeostasis model assessment as a clinical index of insulin resistance. *Diabetes Care* 1999; 22: 1911–1912.
31. 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.
32. Seltzer HS, Allen EW, Herron AL Jr, et al. Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. *J Clin Invest* 1967; 46: 323–335.
33. Rabinowitz D, Zierler KL. Forearm metabolism in obesity and its response to intra-arterial insulin. Characterization of insulin resistance and evidence for adaptive hyperinsulinism. *J Clin Invest* 1962; 41: 2173–2181.
34. DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991; 14: 173–194.
35. Kozawa J, Okita K, Imagawa A, et al. Similar incretin secretion in obese and non-obese Japanese subjects with type 2 diabetes. *Biochem Biophys Res Commun* 2010; 393: 410–413.
36. Lee S, Yabe D, Nohtomi K, et al. Intact glucagon-like peptide-1 levels are not decreased in Japanese patients with type 2 diabetes. *Endocr J* 2009; 57: 119–126.
37. Wishart JM, Horowitz M, Morris HA, et al. Relation between gastric emptying of glucose and plasma concentrations of glucagon-like peptide-1. *Peptides* 1998; 19: 1049–1053.
38. Nielsen S, Guo Z, Johnson CM, et al. Splanchnic lipolysis in human obesity. *J Clin Invest* 2004; 113: 1582–1588.
39. Suzuki H, Fukushima M, Usami M, et al. Factors responsible for development from normal glucose tolerance to isolated postchallenge hyperglycemia. *Diabetes care* 2003; 26: 1211–1215.
40. Madsbad S, Schmitz O, Ransam J, et al. Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. *Diabetes Care* 2004; 27: 1335–1342.
41. Aschner P, Kipnes MS, Luncford JK, et al. Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with Type 2 diabetes. *Diabetes Care* 2006; 29: 2632–2637.
42. Seino Y, Rasmussen MF, Zdravkovic M, et al. 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.
43. Nonaka K, Kakikawa T, Sato A, *et al.* Efficacy and safety of sitagliptin monotherapy in Japanese patients with type 2 diabetes. *Diabetes Res Clin Pract* 2007; 79: 291–298.
44. Mitsui R, Fukushima M, Nishi Y, *et al.* Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men. *Metabolism* 2006; 55: 53–58.
45. Saxena R, Hivert M, Langenberg C, *et al.* Genetic variation in GIPR influences the glucose and insulin responses to an oral glucose challenge. *Nat Genet* 2010; 42: 142–148.

GLP-1 receptor agonist attenuates endoplasmic reticulum stress-mediated β -cell damage in Akita mice

Shunsuke Yamane¹, Yoshiyuki Hamamoto², Shin-ichi Harashima¹, Norio Harada¹, Akihiro Hamasaki¹, Kentaro Toyoda¹, Kazuyo Fujita¹, Erina Joo¹, Yutaka Seino³, Nobuya Inagaki^{1,4*}

ABSTRACT

Aims/Introduction: Endoplasmic reticulum (ER) stress is one of the contributing factors in the development of type 2 diabetes. To investigate the cytoprotective effect of glucagon-like peptide 1 receptor (GLP-1R) signaling *in vivo*, we examined the action of exendin-4 (Ex-4), a potent GLP-1R agonist, on β -cell apoptosis in Akita mice, an animal model of ER stress-mediated diabetes.

Materials and Methods: Ex-4, phosphate-buffered saline (PBS) or phlorizin were injected intraperitoneally twice a day from 3 to 5 weeks-of-age. We evaluated the changes in blood glucose levels, bodyweights, and pancreatic insulin-positive area and number of islets. The effect of Ex-4 on the numbers of C/EBP-homologous protein (CHOP)-, TdT-mediated dUTP-biotin nick-end labeling (TUNEL)- or proliferating cell nuclear antigen-positive β -cells were also evaluated.

Results: Ex-4 significantly reduced blood glucose levels and increased both the insulin-positive area and the number of islets compared with PBS-treated mice. In contrast, there was no significant difference in the insulin-positive area between PBS-treated mice and phlorizin-treated mice, in which blood glucose levels were controlled similarly to those in Ex-4-treated mice. Furthermore, treatment of Akita mice with Ex-4 resulted in a significant decrease in the number of CHOP-positive β -cells and TUNEL-positive β -cells, and in CHOP mRNA levels in β -cells, but there was no significant difference between the PBS-treated group and the phlorizin-treated group. Proliferating cell nuclear antigen staining showed no significant difference among the three groups in proliferation of β -cells.

Conclusions: These data suggest that Ex-4 treatment can attenuate ER stress-mediated β -cell damage, mainly through a reduction of apoptotic cell death that is independent of lowered blood glucose levels. (*J Diabetes Invest*, doi: 10.1111/j.2040-1124.2010.00075.x, 2011)

KEY WORDS: Apoptosis, Endoplasmic reticulum stress, Glucagon-like peptide-1

INTRODUCTION

Type 2 diabetes is a chronic metabolic disorder characterized by the loss of β -cell function and mass. The mechanisms underlying the loss of β -cell function and mass are not fully understood, but recent studies have shown that endoplasmic reticulum (ER) stress is one of the causes of β -cell damage in diabetes¹. Owing to increased demand for insulin secretion, β -cells show a highly developed ER¹. The ER has a number of important functions, such as post-translational modification, folding and assembly of newly synthesized secretory proteins²⁻⁴. Thus, the ER plays an essential role in cell survival. ER function can be impaired by

various conditions, including inhibition of protein glycosylation, reduction in formation of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi and expression of malformed proteins¹. Various physiological or pathological conditions that compromise ER functions are collectively termed ER stress¹⁻³. To alleviate ER stress and promote cell survival, an adaptive response, known as unfolded protein response (UPR) is activated. UPR comprises translational attenuation, induction of chaperones and ER stress-associated degradation (ERAD). However, prolonged activation of UPR can ultimately lead to cell death by apoptosis.

Increased demand for insulin secretion under certain conditions, such as chronic hyperglycemia, might result in β -cell overload. Chronic hyperglycemia in diabetes can therefore induce persistent ER stress, cause β -cell dysfunction and finally lead to a reduction in β -cell mass through apoptosis¹.

Glucagon-like peptide 1 (GLP-1) is a physiological incretin, an intestinal hormone released in response to nutrient

¹Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, ⁴CREST of Japan Science and Technology (JST), Kyoto, ²Center for Diabetes and Endocrinology, Tazuke Kofukai Medical Research Institute, Kitano Hospital, and ³Division of Diabetes, Clinical Nutrition and Endocrinology, Department of Medicine, Kansai Electric Power Hospital, Osaka, Japan

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

Received 21 July 2010; revised 25 August 2010; accepted 8 September 2010

ingestion that stimulates glucose-dependent insulin secretion. A growing body of evidence suggests that GLP-1 not only increases insulin secretion and upregulates insulin biosynthesis, but also stimulates β -cell proliferation and neogenesis⁵⁻⁹, and inhibits β -cell apoptosis⁹⁻¹⁶, resulting in increased β -cell mass. However, demonstration of an *in vivo* effect in the animal models of type 2 diabetes is problematic, because enhancement of GLP-1R signaling lowers blood glucose levels as result of its insulinotropic action, and it is difficult to evaluate the direct cytoprotective effects of GLP-1 in conditions of similar glucose toxicity.

In the present study, we investigated the cytoprotective effect of GLP-1R signaling *in vivo* on ER stress-mediated apoptotic cell death by using Akita mice, an animal model of ER stress-mediated diabetes mellitus. Akita mice have a point mutation in the insulin 2 gene, resulting in misfolding of insulin that leads to severe ER stress^{17,18}. To exclude the possibility that the effect of Ex-4 on β -cells is mediated through improved blood glucose levels, we used three groups of mice: Akita mice treated with phosphate-buffered saline (PBS), Ex-4, or the sodium-coupled glucose transporter inhibitor phlorizin, which decreases blood glucose levels without increasing insulin secretion.

MATERIALS AND METHODS

Experimental Animals

Male C57BL/6 mice and male Akita mice were obtained from Shimizu (Kyoto, Japan). The animals were housed under a light/dark cycle of 12 h with free access to food and water. All experiments were approved by the Kyoto University Animal Care Committee.

In vivo Treatment

The mice were given twice daily intraperitoneal injections of PBS, Ex-4 (24 nmol/kg) or phlorizin (0.3 g/kg) for 2 weeks (from 3 to 5 weeks-of-age). Blood glucose levels were measured every third day by enzyme electrode method using a portable glucose analyzer (Glutest sensor; Sanwakagaku, Nagoya, Japan). Blood samples were collected from tail cuttings from these mice fed *ad libitum*. At the end of the experimental period, blood samples were collected from the inferior vena cava under anesthesia to determine the plasma glycoalbumin levels (Oriental Yeast, Tokyo, Japan). Pancreas samples from each of the animal groups were obtained for histological evaluation, and islets were isolated for measurement of insulin content and RNA extraction.

Evaluation of Pancreatic Insulin-Positive Area and Number of Islets

The pancreas samples were fixed in Bouin's solution. Serial 5- μ m paraffin-embedded tissue sections were mounted on slides. After rehydration, sections were incubated with polyclonal rabbit anti-insulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a biotinylated goat anti-rabbit antibody

(DAKO, Carpinteria, CA, USA), and then with a streptavidin peroxidase conjugate and substrate kit (DAKO) using standard protocols. The total pancreas area and insulin-positive area were quantified on five distal, random, non-overlapping sections from five mice of each group using a BZ-8100 microscope equipped with a BZ-Analyzer (Keyence, Osaka, Japan). Insulin-positive areas and the number of islets of each group were adjusted by total pancreas area¹⁵.

Measurement of Insulin Contents of Isolated Islets

Pancreatic islets were isolated by collagenase digestion. To determine insulin contents, islets were homogenized in 400 μ L acid ethanol (37% HCl in 75% ethanol, 15:1000 [v/v]) and extracted at 4°C overnight. The acidic extracts were dried by vacuum, reconstituted and subjected to insulin measurement. The amount of immunoreactive insulin was determined by radioimmunoassay (RIA).

Measurement of mRNA Expression of C/EBP-Homologous Protein and BiP in Isolated Islets

Measurement of mRNA expression of C/EBP-homologous protein (CHOP) and BiP was carried out by quantitative reverse transcription polymerase chain reaction (RT-PCR) as described previously¹⁹. Briefly, total RNA was extracted from isolated islets with an RNeasy mini kit (Qiagen, Valencia, CA, USA) and treated with DNase (Qiagen). cDNA was prepared by SuperScript Reverse Transcriptase system (Invitrogens, Carlsbad, CA, USA) according to the manufacturer's instructions. CHOP mRNA levels and BiP mRNA levels in the islets were measured by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sequences of forward and reverse primers to evaluate

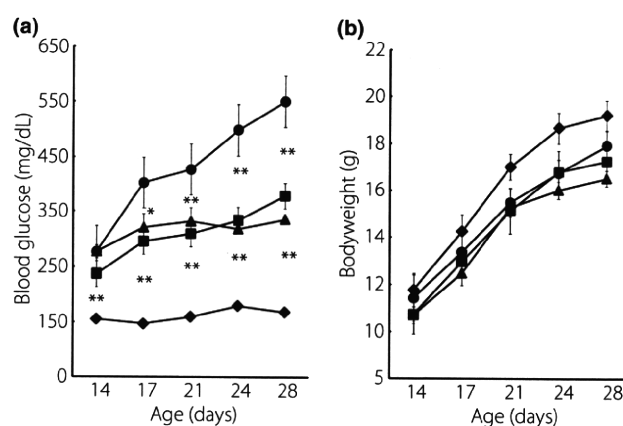


Figure 1 | Ex-4 significantly reduced blood glucose levels in Akita mice. (a) Blood glucose concentration and (b) bodyweight were measured in wild-type C56BL/6 mice (closed diamond, $n = 10$), Akita mice treated with PBS alone (closed circle, $n = 10$), Ex-4 (closed square, $n = 12$) and phlorizin (closed triangle, $n = 10$). Each symbol represents mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$ vs PBS-treated Akita mice.

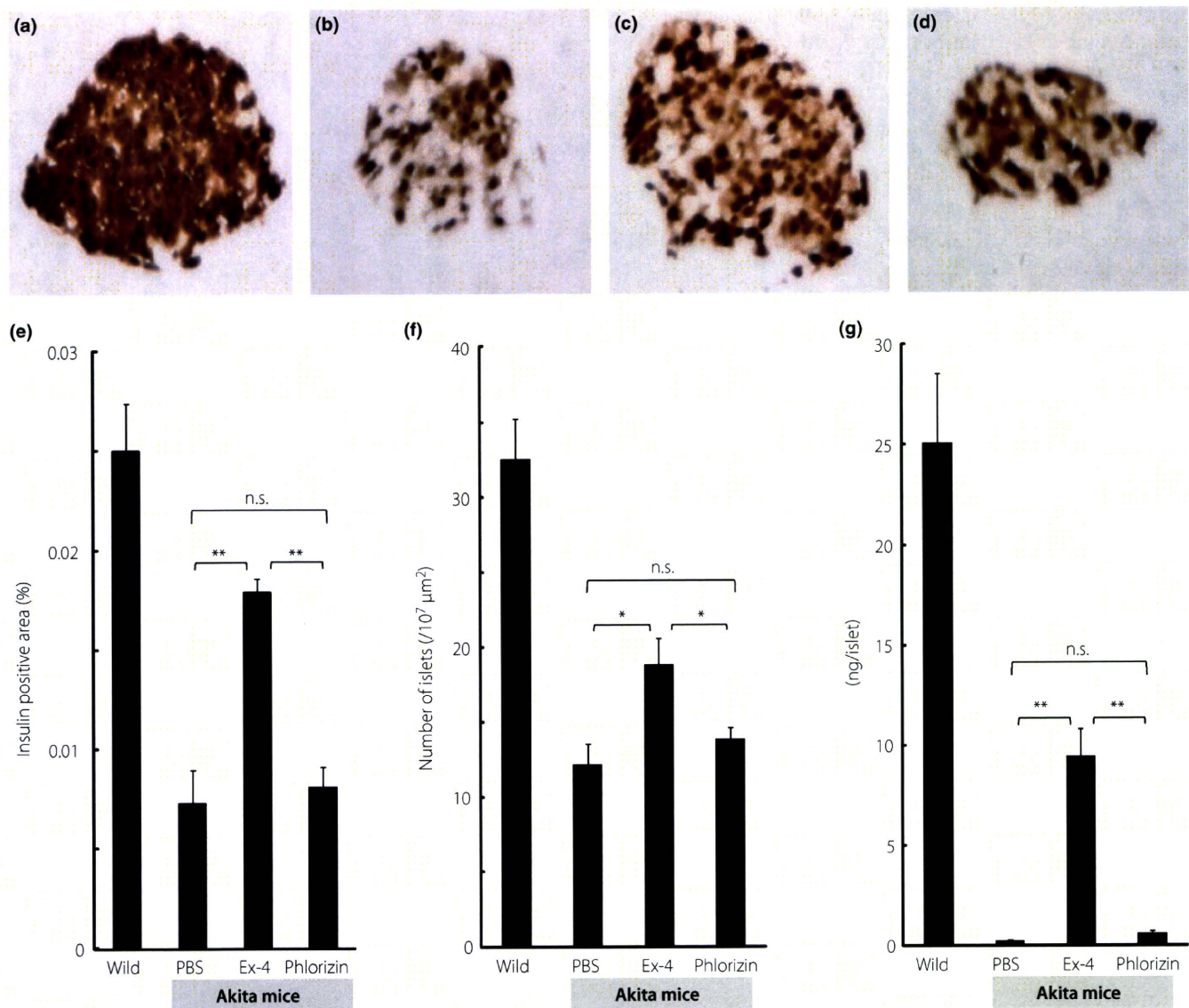


Figure 2 | Ex-4 treatment increased insulin-positive areas, number of islets and insulin content. (a–d) Representative mouse pancreata at 5 weeks-of-age stained with insulin. (a) Wild, (b) Akita mice treated with PBS, (c) Ex-4 or (d) phlorizin. (e) Insulin-positive areas and (f) number of islets were evaluated as described in Materials and Methods ($n = 5$ for each group). (g) Pancreatic insulin content was measured as described in Materials and Methods, and expressed as ng/islet ($n = 5$ for each group). Each column represents mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$.

CHOP expression were 5'-GAGCT- GGAAGCCTGGTATGA-3' and 5'-GGACGCAGGGTCAAGAGTAG-3', respectively; the sequences of forward and reverse primers to evaluate BiP expression were 5'-TTTCTGCCATGGTTCTCACTAA-3' and 5'-GCTGGGCATCATTGAAGTAAG-3', respectively; and the sequences of forward and reverse primers to evaluate glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were 5'-AGCTCACTGGCATGGCTTCCG-3' and 5'-GCCTGCTTCACTTCTTCTTGATG-3', respectively. SYBER Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. Thermal cycling conditions were denatured at 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for

1 min. Total CHOP and total BiP levels were corrected by GAPDH mRNA levels.

Immunofluorescence Staining

For pancreatic CHOP and insulin immunohistochemistry, the tissues were fixed and embedded in paraffin. Serial 5-μm sections were stained with anti-CHOP/GADD153 (Santa Cruz Biotechnology) and anti-insulin (DAKO) antibodies using standard protocols. Insulin immunopositive areas were measured on five distal, random, non-overlapping sections from five mice of each group using a BZ-8100 fluorescence microscope equipped with a BZ-Analyzer (KeyEnce), and the number of cells showing

both nuclear CHOP and cytoplasmic insulin immunopositivity was determined. The ratio of CHOP-positive β -cells was calculated by adjusting the number of CHOP-positive β -cells by the insulin-positive area²⁰. The effect of Ex-4 treatment on β -cell replication and apoptosis was evaluated histologically by proliferating cell nuclear antigen (PCNA) staining (Abcam, Cambridge, MA, USA) and TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining (Takara Bio, Otsu, Japan), respectively. The ratio of TUNEL-positive and PCNA-positive β -cells was also calculated as described earlier.

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical analyses were carried out by unpaired *t*-test. A *P*-value of <0.05 was considered significant.

RESULTS

Effect of Ex-4 on Hyperglycemia and Bodyweight in Akita Mice

Akita mice showed acute and progressive hyperglycemia at 14 days after birth and thereafter. Twice-daily intraperitoneal injection of Ex-4 from 3 to 5 weeks-of-age significantly reduced blood glucose levels compared with those in PBS-treated mice (Figure 1a). Plasma glucose levels in phlorizin-treated Akita mice were similar to those in Ex-4-treated mice. Plasma glycoalbumin levels were significantly lower in the Ex-4- and phlorizin-treated groups than those in the PBS-treated group, but no significant difference was observed between the Ex-4- and phlorizin-treated groups (12.9 ± 1.5 vs 8.7 ± 0.7 vs 8.2 ± 0.6 , respectively, $n = 10$ – 12). Ex-4 treatment or phlorizin treatment did not change bodyweight compared with PBS treatment (Figure 1b). Ex-4 or phlorizin treatment did not change the amount of food intake assessed at 4 weeks-of-age (data not shown).

Effect of Ex-4 on Insulin-Positive Area and Number of Islets

Preservation of β -cell morphology was observed by treatment with Ex-4, as shown in Figure 2a. Quantitative histological analyses showed that Ex-4 treatment significantly increased both the insulin-positive area and the number of islets, whereas there was no significant difference between the PBS-treated group and the phlorizin-treated group (Figure 2b,c).

Effect of Ex-4 on Pancreatic Insulin Content

Figure 2d shows the effect of Ex-4 treatment on insulin content in pancreatic islets. Treatment with Ex-4 significantly increased insulin content in isolated islets, but phlorizin treatment did not.

Quantitative Estimation of CHOP and BiP Expression Levels by Real-Time PCR

The expression levels of CHOP mRNA are shown in Figure 3a, and those of BiP mRNA are shown in Figure 3b. Ex-4 significantly lowered the expression levels of CHOP and BiP mRNA, but there was no significant difference in the expression levels of

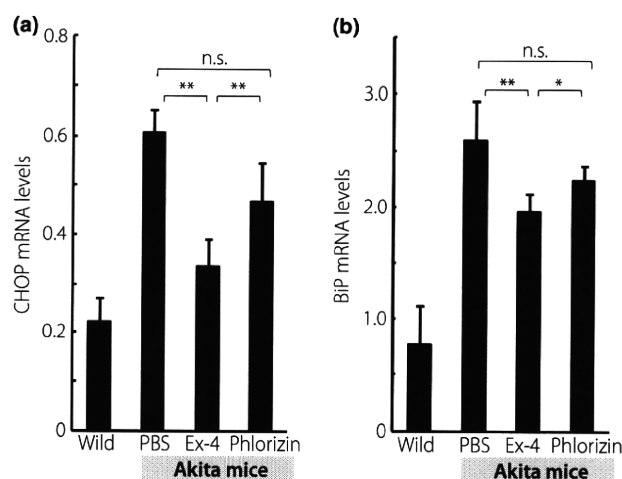


Figure 3 | Ex-4 treatment resulted in a significant decrease in the expression levels of C/EBP-homologous protein (CHOP) mRNA and BiP mRNA in Akita mice. (a) mRNA expression levels of CHOP were evaluated by quantitative real-time polymerase chain reaction (PCR). (b) mRNA expression levels of BiP were evaluated by quantitative real-time PCR. Data are expressed as the ratio to that of glyceraldehyde 3-phosphate dehydrogenase in the same sample ($n = 5$ for each group). Each column represents mean $\pm$ SE. **P* < 0.05, ***P* < 0.01.

CHOP or BiP mRNA between the phlorizin- or PBS-treated groups.

Effect of Ex-4 on the Ratio of CHOP-, TUNEL- and PCNA-Positive β -cells

Figure 4a depicts the representative pancreata stained with insulin (red), CHOP (green) and DAPI (blue), respectively. Similarly, Figure 5a shows the representative pancreata stained with insulin (red) and TUNEL (green). Treatment with Ex-4 significantly decreased the ratio of CHOP-positive β -cells and TUNEL-positive β -cells (Figures 4b and 5b), but there was no significant difference in the ratio of CHOP-positive or TUNEL-positive β -cells between the PBS- and phlorizin-treated groups. Figure 6a shows the representative pancreata stained with insulin (red) and PCNA (green). PCNA staining showed no significant difference in proliferation of β -cells among the three groups of Akita mice (Figure 6b). Interestingly, the ratio of PCNA-positive β -cells was increased in all three groups when compared with wild-type C57BL/6 mice.

DISCUSSION

Akita mice are widely used as an animal model of ER stress-mediated diabetes. Akita mice have a point mutation (C96T) in the insulin 2 gene²¹ that disrupts the disulfide bond formation between the A and B chains of proinsulin, resulting in a drastic conformational change of the molecule. The unfolded proinsulin accumulates to the ER, causing severe ER stress leading to β -cell apoptosis. In humans, it has recently been shown that a mutation in the insulin gene, which is identical

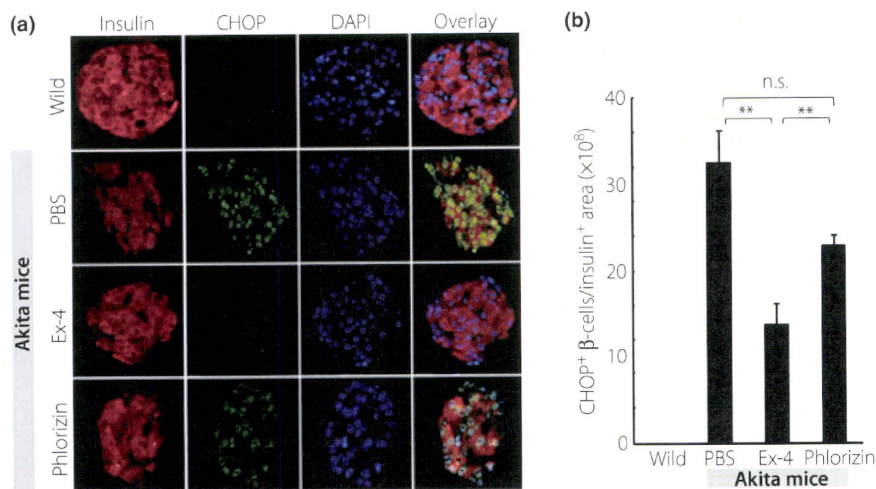


Figure 4 | Ex-4 treatment resulted in a significant decrease in the ratio of C/EBP-homologous protein (CHOP)-positive β-cells in Akita mice. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red), CHOP (green) and DAPI (blue). (b) The number of CHOP-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± SE. ***P* < 0.01.

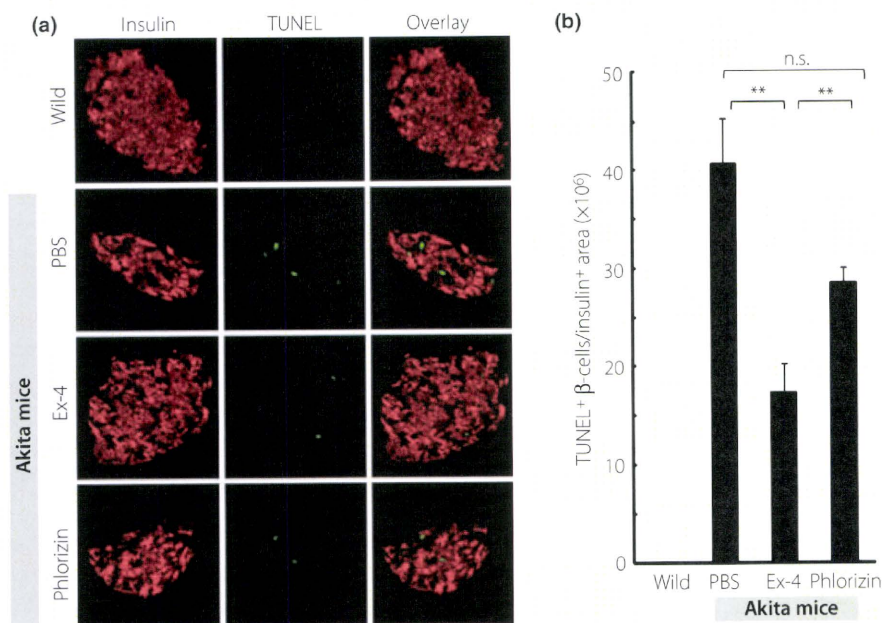


Figure 5 | Ex-4 treatment decreased the ratio of TUNEL-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and TUNEL (green). (b) The number of TUNEL-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± SE. ***P* < 0.01.

to that in the Akita mouse, causes permanent neonatal diabetes within the first month of life that requires lifelong insulin injection²².

In the present study, we have shown that Ex-4 treatment has a protective effect on β-cells in Akita mice. The insulin-positive area and the number of islets were maintained along with a decreased ratio of CHOP- and TUNEL-positive cells in the

islets, showing that the major effect of Ex-4 treatment in the maintenance of β-cell mass is through decreasing β-cell apoptosis in response to ER stress. Because phlorizin decreases blood glucose levels without increasing insulin secretion, it might well reduce ER stress by decreasing the insulin demand. However, in contrast to the Ex-4 treatment, phlorizin treatment failed to show a reduction of ER stress or β-cell protective effects against

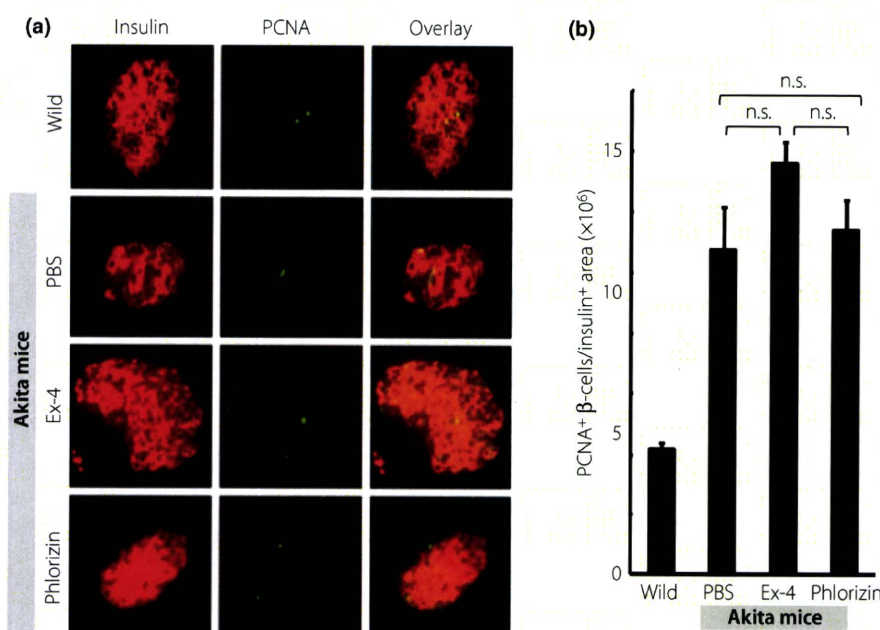


Figure 6 | Ex-4 treatment did not significantly increase the ratio of PCNA-positive β -cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and PCNA (green). (b) The number of PCNA-positive β -cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean $\pm$ SE.

apoptosis in our conditions. These findings show that Ex-4 has a direct effect on ER stress-mediated β -cell apoptosis that is independent of decreased insulin demand.

There are several *in vitro* and *in vivo* studies showing that GLP-1R agonists inhibit β -cell apoptosis^{9–16}, and several molecular mechanisms have been suggested. For example, GLP-1 treatment decreases the expression levels of proapoptotic protein caspase-3 and increases those of anti-apoptotic protein bcl-2 in isolated human islets¹⁰. It also has been shown that the anti-apoptotic effect of Ex-4 is associated with the activation of protein kinase B/Akt through PKA-dependent phosphorylation of CREB¹¹. There are some reports that GLP-1 ameliorates ER stress. Yusta *et al.* found that treatment by Ex-4 reduces blood glucose levels in obese *db/db* mice along with a decrease in the number of CHOP-positive β -cells²⁰. Tsunekawa *et al.*²³ reported a beneficial effect of Ex-4 on β -cell damage in calmodulin-over-expressing transgenic (CaMTg) mice that develop diabetes through ER stress-mediated β -cell apoptosis. They found that Ex-4 treatment reduced blood glucose levels while retaining the insulin-positive areas and decreasing the expression levels of CHOP mRNA in CaMTg mice. *In vitro* studies have found that rapid recovery from translational attenuation¹⁹ or upregulation of BiP and JunB²⁴ accounts for the attenuation of ER stress-mediated β -cell damage by Ex-4 treatment. However, results of chronic Ex-4 treatment in animal models of type 2 diabetes should be carefully interpreted, because enhancement of GLP-1R signaling reduces the blood glucose level by its insulinotropic action. Therefore, the possibility remains that reduced hyperglycemia attenuates persistent ER stress and ameliorates

β -cell apoptosis. Our present findings clearly show that Ex-4 treatment attenuates ER stress-mediated β -cell damage in Akita mice through a reduction of apoptotic cell death that is independent of decreased blood glucose levels.

Although several studies have found that the cytoprotective effect of GLP-1R signaling is not only through inhibition of β -cell apoptosis, but also through stimulation of β -cell proliferation^{5–9}, we did not find any effect of Ex-4 treatment on β -cell proliferation. It is possible that the administration period in the present study was too short to observe β -cell proliferation by Ex-4 or that stimulation of β -cell proliferation does not play a significant role in the cytoprotective effect of GLP-1R signaling in Akita mice. The ratio of PCNA-positive β -cells was increased not only in the Ex-4-treated group of Akita mice, but also in the phlorizin-treated group and the untreated group compared with that in wild-type C57BL/6 mice. Whether or not this result can be attributed to the phenotype of Akita mice requires further study.

Islet mass is reported to be decreased in patients with type 2 diabetes at the time of diagnosis²⁵. Although Ex-4 is in clinical use for treatment of type 2 diabetes²⁶, superiority of Ex-4 over the other antidiabetic drugs has not been shown. Our data confirm the previous findings of a beneficial effect of Ex-4 on glycemic control, but also suggest that Ex-4 has a direct β -cell-protective effect independently of improved glycemic control. Thus, Ex-4 and other GLP-1R agonists might well be more effective than other antidiabetic drugs in clinical use in terms of alleviating β -cell damage and maintaining β -cell mass for diabetic patients.

ACKNOWLEDGEMENTS

This study was supported by Scientific Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and from the Ministry of Health, Labor, Welfare, Japan, and by the Kyoto University Global COE Program 'Center for Frontier Medicine'. There is no conflict of interest for all the authors listed.

REFERENCES

- Oyadomari S, Araki E, Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β -cells. *Apoptosis* 2002; 7: 335–345.
- Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 1999; 13: 1211–1233.
- Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000; 101: 451–454.
- Kaufman RJ, Scheuner D, Schroder M, et al. The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol* 2002; 3: 411–421.
- Friedrichsen BN, Neubauer N, Lee YC, et al. Stimulation of pancreatic β -cell replication by incretins involves transcriptional induction of cyclin D1 via multiple signalling pathways. *J Endocrinol* 2006; 188: 481–492.
- Miettinen P, Ormio P, Hakonen E, et al. EGF receptor in pancreatic β -cell mass regulation. *Biochem Soc Trans* 2008; 36: 280–285.
- Jin T, Liu L. The Wnt signaling pathway effector TCF7L2 and type 2 diabetes mellitus. *Mol Endocrinol* 2008; 22: 2383–2392.
- Zhou J, Pineyro MA, Wang X, et al. Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3 β transcription factors. *J Cell Physiol* 2002; 192: 304–314.
- Wang Q, Brubaker PL. Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* 2002; 45: 1263–1273.
- Farilla L, Bulotta A, Hirshberg B, et al. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology* 2003; 144: 5149–5158.
- Jhala US, Canettieri G, Screaton RA, et al. cAMP promotes pancreatic β -cell survival via CREB-mediated induction of IRS2. *Genes Dev* 2003; 17: 1575–1580.
- Li Y, Hansotia T, Yusta B, et al. Glucagon-like peptide-1 receptor signaling modulates β cell apoptosis. *J Biol Chem* 2003; 278: 471–478.
- Buteau J, El Assaad W, Rhodes CJ, et al. Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia* 2004; 47: 806–815.
- Bregenholt S, Moldrup A, Blume N, et al. The long-acting glucagon-like peptide-1 analogue, liraglutide, inhibits β -cell apoptosis *in vitro*. *Biochem Biophys Res Commun* 2005; 330: 577–584.
- Park S, Dong X, Fisher TL, et al. Exendin-4 uses Irs2 signaling to mediate pancreatic β cell growth and function. *J Biol Chem* 2006; 281: 1159–1168.
- Toyoda K, Okitsu T, Yamane S, et al. GLP-1 receptor signaling protects pancreatic beta cells in intraportal islet transplant by inhibiting apoptosis. *Biochem Biophys Res Commun* 2008; 367: 793–798.
- Yoshioka M, Kayo T, Ikeda T, et al. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 1997; 46: 887–894.
- Oyadomari S, Koizumi A, Takeda K, et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 2002; 109: 525–532.
- Harada N, Yamada Y, Tsukiyama K, et al. A novel GIP receptor splice variant influences GIP sensitivity of pancreatic β -cells in obese mice. *Am J Physiol Endocrinol Metab* 2008; 294: E61–E68.
- Yusta B, Baggio LL, Estall JL, et al. GLP-1 receptor activation improves β cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* 2006; 4: 391–406.
- Wang J, Takeuchi T, Tanaka S, et al. A mutation in the insulin 2 gene induces diabetes with severe pancreatic β -cell dysfunction in the Mody mouse. *J Clin Invest* 1999; 103: 27–37.
- Stoy J, Edghill EL, Flanagan SE, et al. Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc Natl Acad Sci USA* 2007; 104: 15040–15044.
- Tsunekawa S, Yamamoto N, Tsukamoto K, et al. Protection of pancreatic β -cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; *in vivo* and *in vitro* studies. *J Endocrinol* 2007; 193: 65–74.
- Cunha DA, Ladriere L, Ortis F, et al. Glucagon-like peptide-1 agonists protect pancreatic β -cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* 2009; 58: 2851–2862.
- Butler AE, Janson J, Bonner-Weir S, et al. β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003; 52: 102–110.
- Kendall DM, Riddle MC, Rosenstock J, et al. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. *Diabetes Care* 2005; 28: 1083–1091.

Three-dimensional *ex vivo* imaging and analysis of intraportal islet transplants

Hiroyuki Fujimoto ^a, Kentaro Toyoda ^a, Teru Okitsu ^b, Xibao Liu ^{a, b}, Eri Mukai ^a,
Xiaotong Zhuang ^a, Shinji Uemoto ^c, Naoki Mochizuki ^d and Nobuya Inagaki ^{a, c *}

^a Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan

^b Transplantation Unit, Kyoto University Hospital, Kyoto, Japan

^c Department of Surgery, Division of Hepato-pancreato-biliary surgery and Transplantation, Graduate School of Medicine, Kyoto University, Kyoto, Japan

^d Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan

^e CREST of Japan Science and Technology Cooperation (JST), Kyoto, Japan

Authorship

Each author's contribution or role

Hiroyuki Fujimoto; designed study, performed study, collected data, analyzed data, wrote paper

Kentaro Toyoda; designed study, performed study, wrote paper

Teru Okitsu; designed study, Xibao Liu; performed study, Eri Mukai; performed study

Xiaotong Zhuang; performed study, Shinji Uemoto; designed study,

Naoki Mochizuki; designed study, Nobuya Inagaki; designed study, wrote paper

All authors have no conflict of interest.

Funding

This work was supported by a Research Grant from the Ministry of Health, Labour, and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and also by Kyoto University Global COE Program “Center for Frontier Medicine”.

*To whom correspondence should be addressed.

54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Tel: +81-75-751-3560, Fax: +81-75-751-4244.

E-mail address: inagki@metab.kuhp.kyoto-u.ac.jp (N. Inagaki)

ABSTRACT

In clinical islet transplantation, because the long-term insulin-independence rate is still poor, a method for detailed analysis of the transplanted islets in the liver after transplantation is required. We have established a novel imaging technique suitable for analysis of transplanted islets in liver using an optical projection tomography (OPT) method. A three-dimensional tomographic image of the transplanted islets in liver was reconstructed. The number of islets transplanted and the number of transplanted islets observed by OPT showed good correlation. The OPT method was used to compare the numbers of transplanted islets in mouse syngeneic and allogeneic transplantation models. Blood glucose concentrations of STZ-induced diabetic mice transplanted with syngeneic islets remained normoglycemic and the number of transplanted islets was largely preserved 11 days after transplantation. In mice transplanted with allogeneic islets, hyperglycemia recurred from 7 days after transplantation, and the number and the volume of transplanted islets was significantly reduced 11 days after transplantation. These results indicate that OPT imaging and analysis may be a useful tool to quantitatively and sterically evaluation of transplanted islets in liver at the cellular level.

KEYWORDS; Optical Projection Tomography, Islet transplantation, three dimensional images, syngeneic transplantation, allogeneic transplantation

Introduction

Islet transplantation is a promising therapeutic approach for patients with insulin-dependent diabetes mellitus to achieve insulin-independence (1, 2). A remarkably high rate of freedom from insulin therapy is achieved in insulin-dependent type 1 diabetic patients after islet transplantation by the Edmonton Protocol (1). However, it was reported that long-term maintenance of glucose homeostasis without the use of insulin is poor (3). This decline may be due to progressive islet loss as well as to various reactions during and after islet transplantation, including mechanical injury, ischemia, and non-specific inflammatory reactions (4).

Until now, the total functional volume of islets transplanted intraportally in liver could be monitored only indirectly by measurements such as blood glucose and serum c-peptide levels. Modalities including bioluminescence imaging (BLI) (5-7), magnetic resonance imaging (MRI) (8-11), and positron emission tomography (PET) (12-14) have been used, and transplanted islets were detected by MRI (11) and PET (13) in human. These methods are suitable for in vivo examination because they are non-invasive and can be repeated over time. However, islets cannot be evaluated at the cellular level by these methods because the resolution is too low. Conventional immunohistochemical method permits evaluation of beta-cell volume at the sub-cellular level, but can observe only restricted, sliced areas of the sample. Recently, Hara M et al. reported sub-cellular analysis of intact pancreas, but the method can analyze only thin neonatal samples (15). To investigate engraftment of transplanted islets scattered in solid liver at the subcellular level, another method is required. We have demonstrated an OPT technique for precisely, three-dimensionally evaluating transplanted islets at the cellular level in liver.