Table 2 | Clinical profiles of patients who achieved good glycemic control without requiring the use of insulin and those requiring insulin to achieve good glycemic control

	Non-insulin	Insulin	*P*
No. subjects	119	82	
Male/female	82/37	45/37	
Age (years)	58.4 ± 1.1	62.9 ± 1.3*	0.0099
Systolic blood pressure (mmHg)	124.4 ± 1.4	126.4 ± 1.7	0.3598
Diastolic blood pressure (mmHg)	77.3 ± 1.0	73.3 ± 1.3	0.0135
BMI (kg/m^2)	26.0 ± 0.4	24.0 ± 0.4*	0.0019
HbA_{1c} at admission (%)	9.2 ± 0.2	10.0 ± 0.2*	0.0050
PG at admission (mg/dL)	163.2 ± 5.0	206.9 ± 8.0*	<0.0001
PG at discharge (mg/dL)	110.9 ± 1.2	114.2 ± 1.3	0.0602
Years from diagnosis	7.8 ± 0.6	10.9 ± 1.0*	0.0052
FCPR (ng/mL)	2.06 ± 0.07	1.61 ± 0.09*	0.0001
CPR-6 min (ng/mL)	4.48 ± 0.18	3.29 ± 0.19*	<0.0001
ΔCPR (ng/mL)	2.43 ± 0.12	1.68 ± 0.12*	<0.0001
SUIT (%)	47.2 ± 2.5	31.1 ± 2.7*	<0.0001
CPI (ng/mg)	1.57 ± 0.07	1.06 ± 0.06*	<0.0001

Data are presented as mean ± SE. *P < 0.01 versus non-insulin. Good glycemic control: mean preprandial capillary plasma glucose levels at discharge <130 mg/dL.

BMI, body mass index; CPI, C-peptide index; ΔCPR, increment of C-peptide immunoreactivity; CPR-6 min, C-peptide immunoreactivity 6 min after intravenous injection of glucagon; FCPR, fasting C-peptide immunoreactivity; PG, mean preprandial capillary plasma glucose level; SUIT, secretory unit of islet in transplantation index.

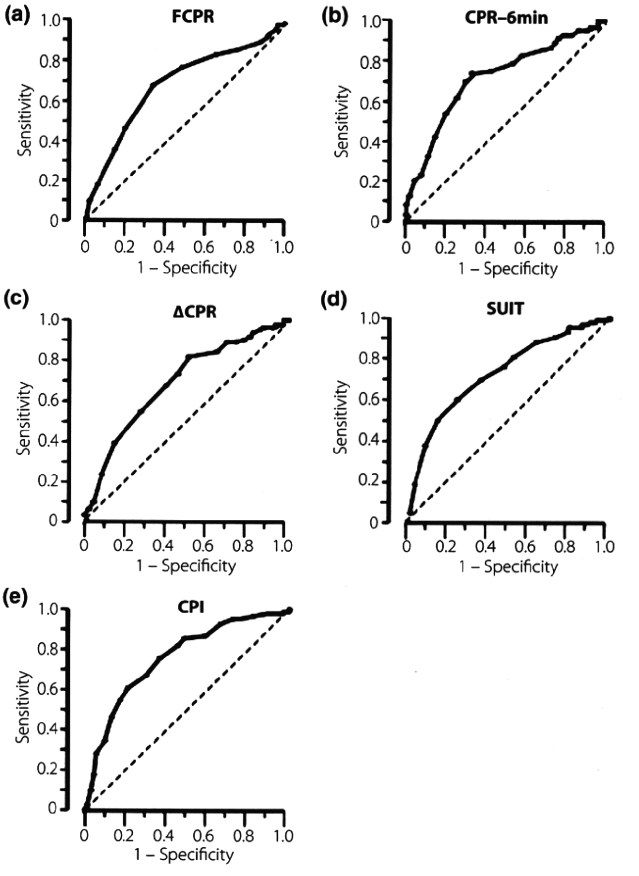

Figure 1 | Receiver–operator characteristic curves of (a) fasting C-peptide immunoreactivity (FCPR), (b) CPR 6 min after intravenous injection of glucagon (CPR-6 min), (c) increment of CPR (ΔCPR), (d) secretory unit of islet in transplantation index (SUIT) and (e) C-peptide index (CPI) of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge.

respectively in almost all (more than 95%) patients. Daily insulin dosage was 22.0 ± 11.1 U (mean ± SD) in the insulin group.

In Figure S3, peak relative frequency of indices using CPR of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge in the insulin group and the non-insulin group, respectively, is shown (FCPR: 1.50–1.75, 2.00–2.25 ng/mL; CPR-6 min: 2.75–3.00, 4.00–4.25 ng/mL; ΔCPR: 1.25–1.50, 1.25–1.50 plus 2.25–2.50 ng/mL; SUIT: 15–20, 25–30 plus 35–40 plus 45–50%; and CPI: 0.8–0.9, 1.5–1.6 ng/mg). According to ROC curves of indices using CPR shown in Figure 1, AUC, cut-off values and values at optimal cut-off points including sensitivity, specificity and the likelihood ratio were determined and shown in Table 3. CPI is the most relevant of these indices for selecting insulin therapy to achieve good glycemic control, because the likelihood ratio and AUC of CPI is greatest.

The ROC curve of CPI of patients who achieved <7.4% HbA_{1c} within 6 months after discharge is shown in Figure 2. According to ROC curves of CPI in Figure 2, the AUC (0.75), cut-off values (optimal: 1.2; 90% specificity 0.8; 90% sensitivity 1.7 ng/mg), and values at optimal cut-off points including sensitivity (73%), specificity (71%) and the likelihood ratio (2.5) were determined.

DISCUSSION

Medical nutritional therapy (MNT) improves glycemic control in patients with type 2 diabetes regardless of their modes of therapy including diet alone, OHA and insulin[22–24]. Diet therapy is the basis and starting point of treatment of all patients with diabetes[25], and failure of diet therapy alone might predict the inability to attain optimal glycemic control by any of these modes of therapy. To precisely analyze the relationship between endogenous insulin secretion and the appropriate mode of therapy for achieving good glycemic control, we used data of hospitalized patients under optimal therapy including proper MNT. Thus, our results are more likely to be valid in patients with appropriate care behaviors. Although inappropriate care behavior is an obstacle to achieving good glycemic control over a longer duration, our results suggest a basis for beginning insulin therapy in patients who do not achieve good glycemic control with diet alone or OHA despite the practice of appropriate care behavior.

Table 3 | Analysis of indices using serum C-peptide of patients with mean preprandial capillary plasma glucose levels of <130 mg/dL at discharge

	FCPR	CPR-6 min	ΔCPR	SUIT	CPI
AUC	0.69	0.71	0.69	0.72	0.75
Cut-off values	(ng/mL)	(ng/mL)	(ng/mL)	(%)	(ng/mg)
Optimal	1.75	3.75	2.25	30	1.1
90% Specificity	1.00	2.25	1.00	20	0.7
90% Sensitivity	2.75	5.25	3.25	55	1.7
Values at optimal cut-off points					
Sensitivity (%)	70	74	82	61	61
Specificity (%)	66	65	49	73	78
Likelihood ratio	2.0	2.1	1.6	2.3	2.8

AUC, area under receiver–operator characteristics curve; CPI, C-peptide index; ΔCPR, increment of C-peptide immunoreactivity; CPR-6 min, C-peptide immunoreactivity 6 min after intravenous injection of glucagon; FCPR, fasting C-peptide immunoreactivity; SUIT, secretory unit of islet in transplantation index

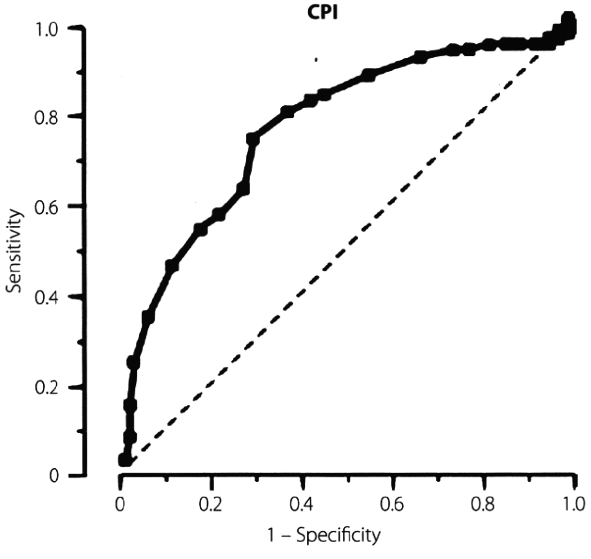

Figure 2 | Receiver–operator characteristic curve of C-peptide index (CPI) of patients who achieved <7.4% HbA_{1c} within 6 months after discharge.

In the present study, just 42% of patients achieved good control during hospital admission, partly because the aim of admission was not necessarily to achieve good control during the period of admission, but to establish a treatment policy for the achievement of good control after discharge. The percentage of patients treated with insulin at discharge was higher in the non-achieved group than in the achieved group (non-achieved group: 67%; achieved group: 41%). Of the patients treated with OHA at admission in the achieved group, 39% had therapy changed to insulin, whereas 73% of the patients treated with OHA at admission in the non-achieved group had therapy changed to insulin. These results might indicate more intensive therapy in the case of the non-achieved group. Of the 136 patients in the non-achieved group at discharge, 76 showed <7.4% HbA_{1c} within 6 months after discharge, showing fair glycemic control in some of the patients of this group over the longer term. As shown in Table 1, the non-achieved group had more progressive diabetic complications and more years from diagnosis compared with the achieved group. These factors might prompt therapy that aims at a more gradual improvement of glycemic control to prevent hypoglycemia. In addition, the non-achieved group showed higher glycemic levels at admission than that of the achieved group, whereas the duration of hospitalization was similar.

Although there have been several reports regarding the utility of indices of endogenous insulin secretion to indicate initiation of insulin therapy to improve glycemic control[11–14], none has compared the utility of the various indices. In the present study, as shown by the likelihood ratio and by AUC, CPI is shown to be the most useful among the five indices.

CPI was used as an index of endogenous insulin secretion in several reports[26–28], but its advantage over other indices and the scientific basis was unclear. The SUIT index (SUIT) was developed using FCPR and plasma glucose level after islet transplantation[19]. The linear relationship between FCPR and FPG in individual subjects shows a plasma glucose level (61.7 mg/dL) assumed to suppress C-peptide to zero. Transplantation of islets from non-diabetic donors increases the slope (FCPR/[FPG – 61.7]), suggesting an index of transplanted β-cell mass. Although a correlation between SUIT and CPR 6 min after intravenous injection of 1 mg glucagon (CPR-6 min) is observed in type 2 diabetes ($r = 0.58$), it is weaker than that in patients after islet transplantation ($r = 0.82$)[19].

Autopsy reveals that β-cell mass is decreased in patients with type 2 diabetes compared with that in healthy subjects[29–31]. Recently, in 33 subjects at various stages of glucose tolerance, a correlation between β-cell areas of a sample obtained during pancreatectomy, and serum levels of CPR and insulin before the operation was analyzed[32]. Interestingly, β-cell areas are positively correlated with fasting insulin/FPG ($r = 0.51$, $P = 0.0024$) and FCPR/FPG ($r = 0.63$, $P < 0.0001$), but are not significantly

correlated with homeostasis model assessment β-cell function (HOMA-β). Because SUIT 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 SUIT in subjects with glucose intolerance. Furthermore, CPI is not affected by exogenous insulin[27], 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 inadequate[14]. 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.

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The authors declare no conflict of interest.

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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

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

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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.
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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 secretion[1]. 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 compared 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 diabetes[10]. In studies using rodent models, it was reported that GIPR mRNA and protein expression levels in islets are decreased in the diabetic state[11]. In contrast, in the non-diabetic obese state, GIP plays an important role in maintaining blood glucose levels[12]. 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 mice[16]. 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

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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 subjects[23]. 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/dL[24]. 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 recommendations[25]. NGT was diagnosed according to World Health Organization (WHO) criteria[26].

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 index[31] and insulin sensitivity index (ISI) composite[32]. The calculations of the four indices were as follows:

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

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

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

$$\text{IRI composite} = 10{,}000 / (\text{FGP [mg/dL]} \times \text{FIRI [μU/mL]} \times \text{mean OGTT PG [mg/dL]} \times \text{mean OGTT IRI [μU/mL]})^{0.5}$$

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 ± 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/m^2. No subjects had liver or kidney dysfunction. HbA_{1c}, FPG, TG, total

Table 1 | Clinical characteristics of the subjects

n (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. 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-cells[11], whereas GIP signaling is enhanced and maintains

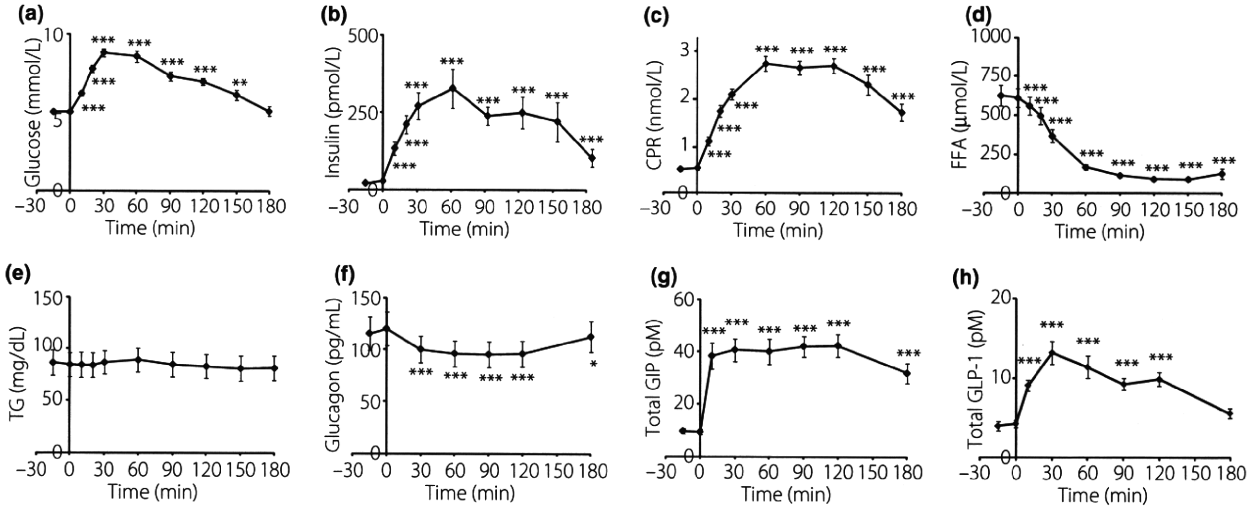

Figure 1 | Concentrations of (a) plasma glucose, (b) serum immunoreactive insulin, (c) serum C-peptide reactivity (CPR), (d) serum free fatty acid (FFA), (e) serum triglyceride (TG), (f) glucagon, (g) total gastric inhibitory polypeptide (GIP) and (h) total glucagon-like peptide-1 (GLP-1) during oral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

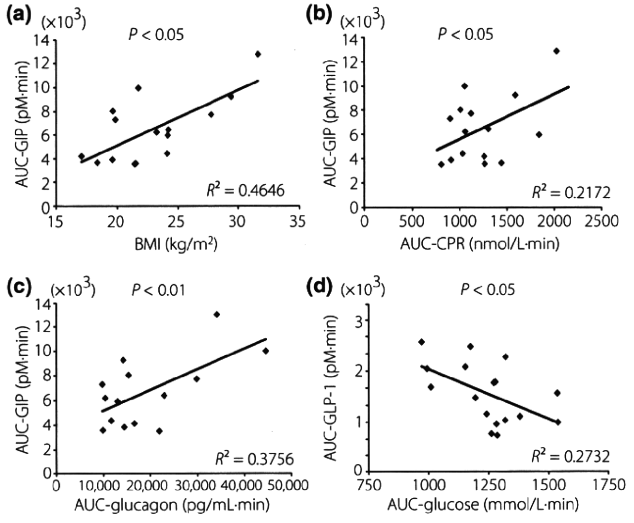

Figure 2 | Simple regression analysis of gastric inhibitory polypeptide secretion (AUC-GIP) and (a) body mass index (BMI), (b) AUC of serum C-peptide reactivity (CPR) and (c) glucagon. (d) Simple regression analysis of glucagon-like peptide-1 secretion (AUC-GLP-1) and AUC of plasma glucose (PG).

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 OGTT[18]. 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 loading[1], 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

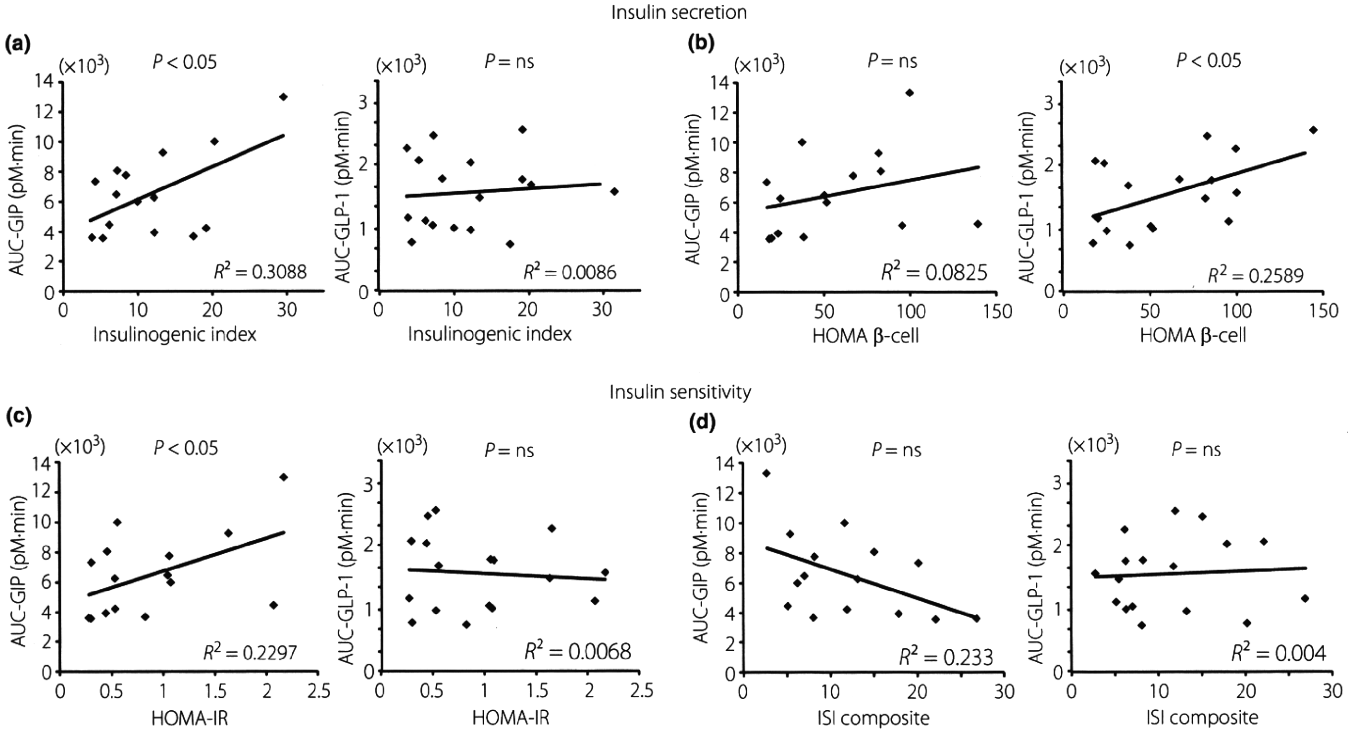

Figure 3 | Relationship between gastric inhibitory polypeptide secretion (AUC-GIP) and glucagon-like peptide-1 secretion (AUC-GLP-1) and the indices of insulin secretion and insulin sensitivity. (a) Insulinogenic index, (b) homeostasis model assessment (HOMA) β-cell function, (c) homeostasis model assessment 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 empting in Caucasian subjects[37]. Although we did not measure gastric empting 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 subjects[38]. However, because Japanese subjects are less obese than Caucasian subjects[21], 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 secretion[1], 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 diabetes[39], 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 patients[44]. 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 studies[45]. 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.

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

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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 diabetes[1]. Owing to increased demand for insulin secretion, β-cells show a highly developed ER[1]. The ER has a number of important functions, such as post-translational modification, folding and assembly of newly synthesized secretory proteins[2–4]. 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 malfolded proteins[1]. Various physiological or pathological conditions that compromise ER functions are collectively termed ER stress[1–3]. 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 apoptosis[1].

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

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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 neogenesis[5–9], and inhibits β-cell apoptosis[9–16], 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-μm 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 area[15].

Measurement of Insulin Contents of Isolated Islets

Pancreatic islets were isolated by collagenase digestion. To determine insulin contents, islets were homogenized in 400 μL 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 previously[19]. 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

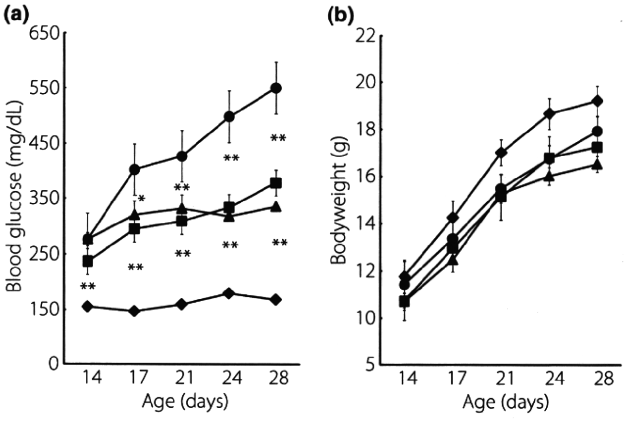

Figure 1 | Ex-4 significantly reduced blood glucose levels in Akita mice. (a) Blood glucose concentration and (b) bodyweight were measured in wild-type C56BL/6 mice (closed diamond, $n = 10$), Akita mice treated with PBS alone (closed circle, $n = 10$), Ex-4 (closed square, $n = 12$) and phlorizin (closed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

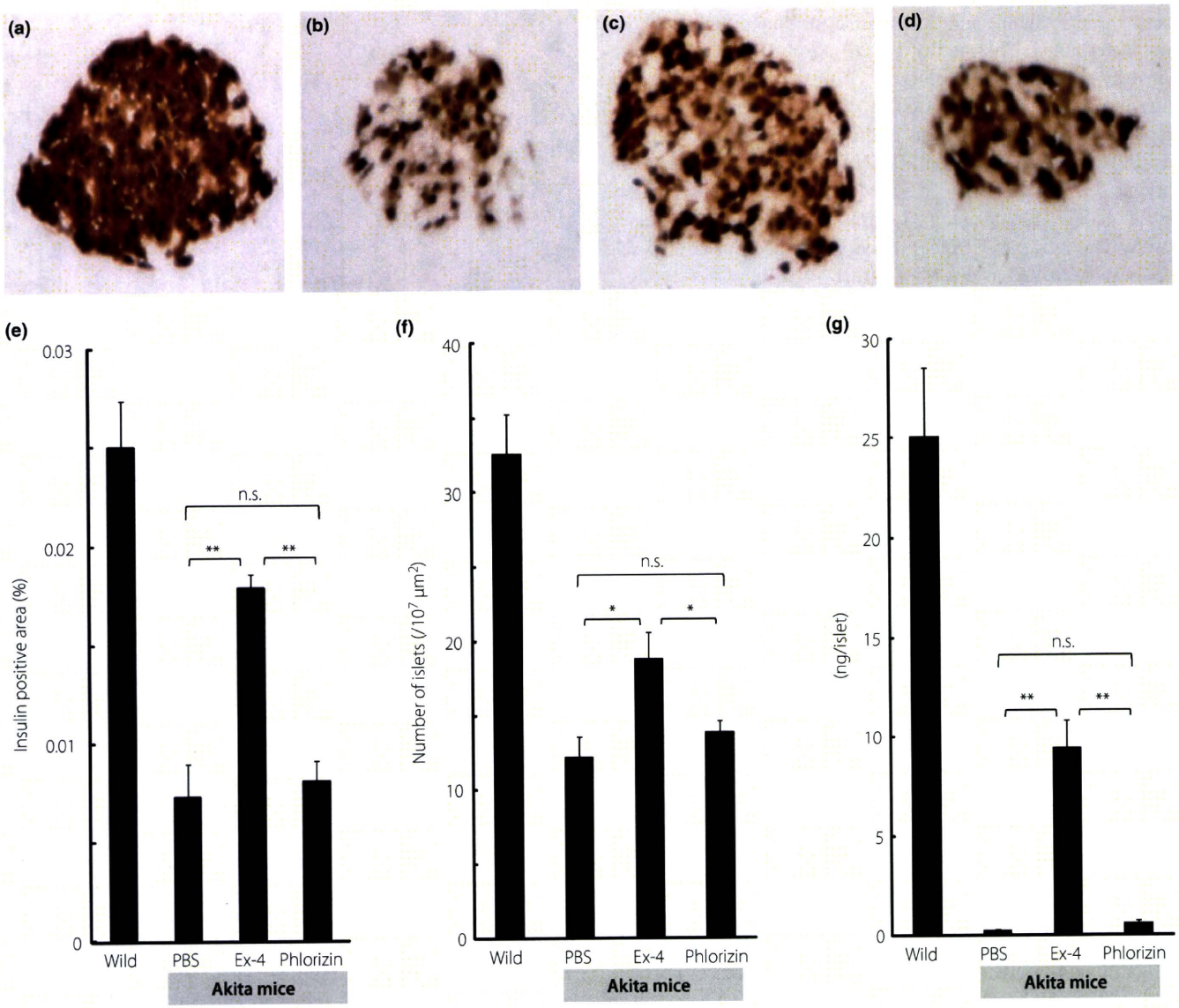

Figure 2 | Ex-4 treatment increased insulin-positive areas, number of islets and insulin content. (a–d) Representative mouse pancreata at 5 weeks-of-age stained with insulin. (a) Wild, (b) Akita mice treated with PBS, (c) Ex-4 or (d) phlorizin. (e) Insulin-positive areas and (f) number of islets were 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 ± 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′-GCCTGCTTCACCACCTTCTTGATG-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 area[20]. 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 ± 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 CHOP or BiP mRNA between the phlorizin- or PBS-treated groups.

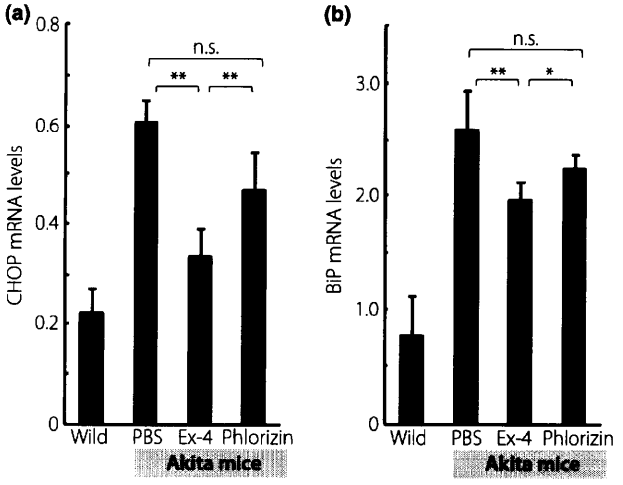

Figure 3 | Ex-4 treatment resulted in a significant decrease in the expression levels of C/EBP-homologous protein (CHOP) mRNA and Bip mRNA in Akita mice. (a) mRNA expression levels of CHOP were evaluated by quantitative real-time polymerase chain reaction (PCR). (b) mRNA expression levels of BiP were 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 ± SE. *P < 0.05, **P < 0.01.

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 gene[21] 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

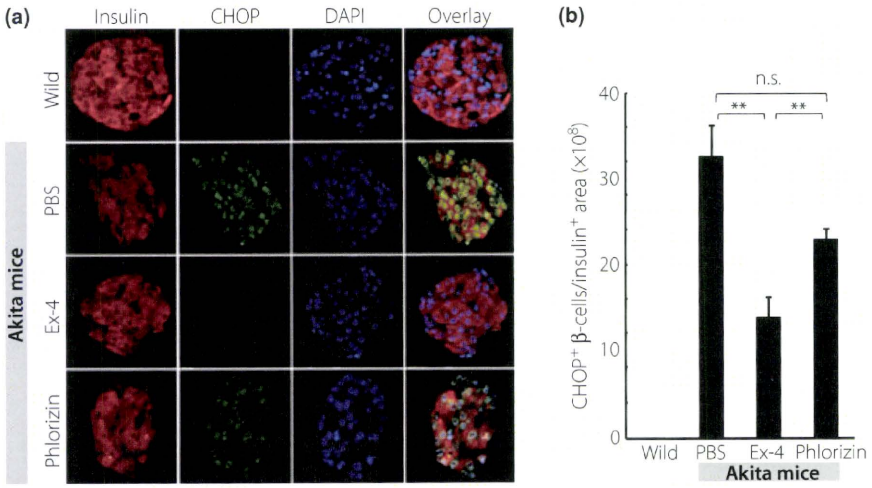

Figure 4 | Ex-4 treatment resulted in a significant decrease in the ratio of C/EBP-homologous protein (CHOP)-positive β-cells in Akita mice. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red), CHOP (green) and DAPI (blue). (b) The number of CHOP-positive β-cells normalized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

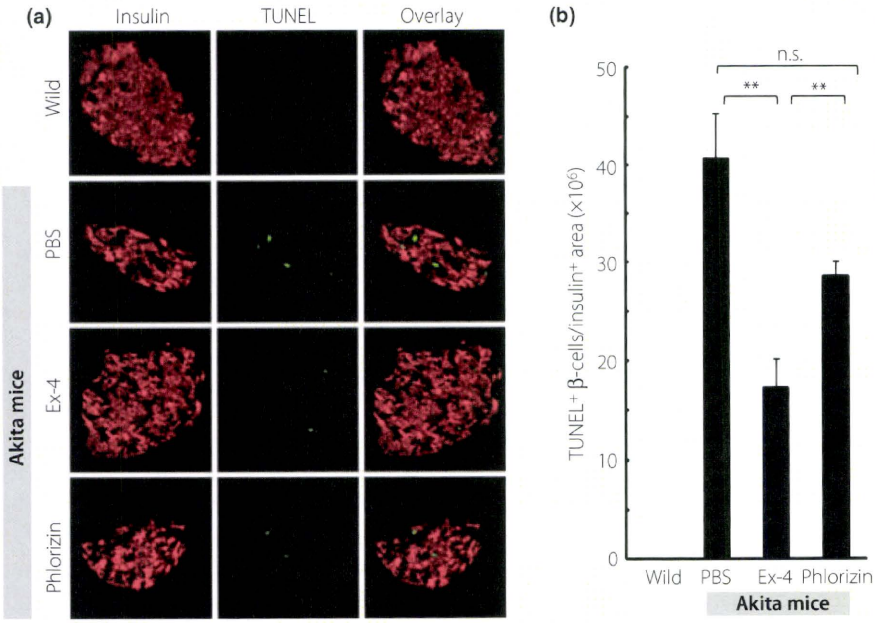

Figure 5 | Ex-4 treatment decreased the ratio of TUNEL-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and TUNEL (green). (b) The number of TUNEL-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. 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 injection[22].

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

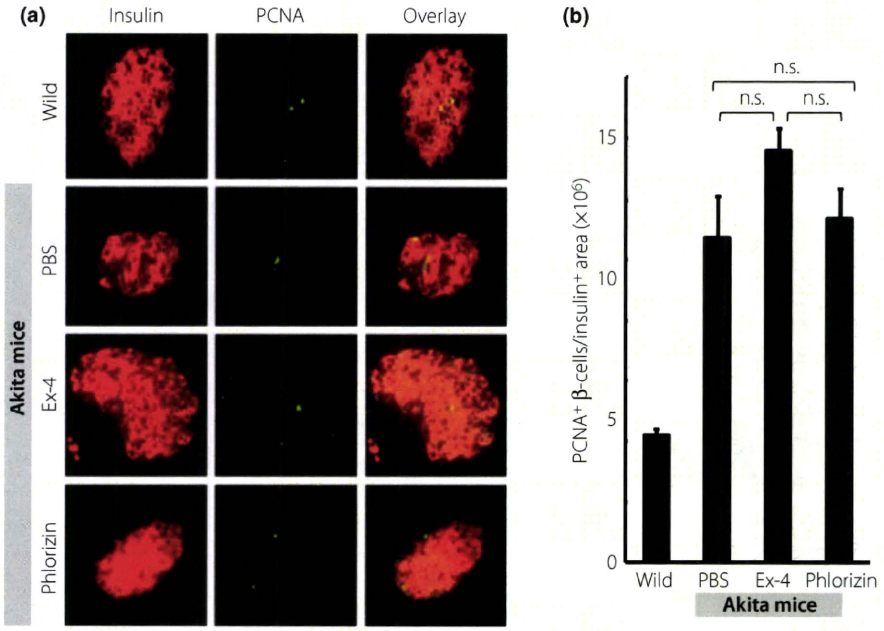

Figure 6 | Ex-4 treatment did not significantly increase the ratio of PCNA-positive β-cells. (a) Representative mouse pancreata at 5 weeks-of-age stained with insulin (red) and PCNA (green). (b) The number of PCNA-positive β-cells normalized per insulin-positive area was quantified as described in Materials and Methods. Each column represents mean ± 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 islets[10]. 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 CREB[11]. 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-cells[20]. 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 attenuation[19] or upregulation of BiP and JunB[24] 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 diagnosis[25]. Although Ex-4 is in clinical use for treatment of type 2 diabetes[26], 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.

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

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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

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