IL8 real-time qPCR

Total RNA was extracted using a nucleospin extract II kit (Macherey Nagel, Duren, Germany). Reverse transcription was performed with 0.8 μg of total extracted RNA, using the ABI high-capacity cDNA archive kit (Applied Biosystems). RT-PCR was performed using an ABI StepOnePlus™. Each reaction contained 10 μl of 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), 1 μl of IL-8 (Hs00174103_m1), ABCA3 (Hs00975518_m1) or GAPDH (Hs03929097_g1) TaqMan® probe and 40 ng of cDNA as the template in a final volume of 20 μl. Data were analyzed using the comparative C_t method ($\Delta\Delta C_t$). For relative quantification, the amount of IL-8 was normalized for GAPDH (endogenous gene) relative to wild-type cells (ABCA3-WT) used as the calibrator and was calculated using the $2^{-\Delta\Delta C_t}$ method as published previously (35). Each point corresponds to the mean ± SD of three experiments performed in triplicate.

Western blot

BALF proteins were accurately quantified using a Qubit fluorometer (Invitrogen). Then, 24 μg of protein was fractionated using SDS–PAGE on 16% Tris–tricine gels, electrotransferred and probed by immunoblotting using antibodies to surfactant proteins SP-B and SP-C (Seven Hills Bioreagents, Cincinnati, OH, USA), as described previously (33).

A549 cell extracts were prepared from 3×10^5 cells and solubilized as described previously (34). An equal amount of protein (10 μg) from each sample was size-separated on 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Immunodetection was performed with antibodies specific for the total and phosphorylated forms of ERK1/2 (Cell Signaling Technology, Beverly, MA, USA) and β-actin (Sigma-Aldrich). Secondary antibodies were from Cell Signaling Technology. Bound antibodies were detected using Super-Signal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Between successive probes, membranes were treated with Restore Western Blot Stripping Reagent (Pierce). Molecular masses were determined using the SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). Images were recorded with a Fujifilm LAS-3000 bioimaging system (Fujifilm, Stamford, CT, USA).

For the study of ABCA3 expression, 35 μg of transiently transfected cells (Lipofectamin, 48 h) was used. Immunoblotting was performed with an anti-eGFP antibody (Clontech, Mountain View, CA, USA).

Statistics

The statistical significance of differences between groups was tested using the unpaired Student's *t*-test with a threshold of $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Analysis of factors influencing postprandial C-peptide levels in Japanese patients with type 2 diabetes: Comparison with C-peptide levels after glucagon load

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ABSTRACT

Aims/Introduction: Postprandial serum C-peptide levels are readily determined in clinical practice and have a good correlation with serum C-peptide levels after glucagon load; the measurement is often used as an index of endogenous insulin secretion. However, the factors affecting postprandial serum C-peptide levels remain to be evaluated.

Materials and Methods: To investigate the clinical factors affecting postprandial serum C-peptide, 2-h postprandial C-peptide levels after breakfast (PPCPR) were analyzed retrospectively for comparison with glucagon-stimulated C-peptide (CPR-6min) levels measured during hospital admission in 273 Japanese patients with type 2 diabetes.

Results: Multiple regression analysis showed that years from diagnosis, body mass index (BMI) and HbA_{1c} were the major independent variables predicting PPCPR ($R^2 = 0.315$). HbA_{1c} was a major factor predicting PPCPR, but did not predict CPR-6min. In addition, HbA_{1c} was negatively correlated with PPCPR ($r = -0.410$, $P < 0.0001$) and PPCPR/CPR-6min ($r = -0.313$, $P < 0.0001$).

Conclusions: PPCPR was correlated with common factors predicting CPR, including years from diagnosis and BMI, but also was negatively correlated with HbA_{1c}, a unique factor. These results show that chronic elevation of the glucose level might impair endogenous insulin secretion after meal load, but might have little effect on endogenous insulin secretion after glucagon load.
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KEY WORDS: C-peptide, Meal load, HbA_{1c}

INTRODUCTION

Type 2 diabetes is a heterogeneous disease characterized by insulin resistance and defective insulin secretion[1], and is progressive in that the mode of therapy must be altered over the decades of diabetes; diet and exercise therapy alone might be adequate initially, but secondary oral hypoglycemic agent (OHA) treatment and insulin treatment are eventually required[2,3]. This is, at least in part, as a result of progressive loss of pancreatic β-cell function. The results of the United Kingdom Progressive Diabetes Study (UKPDS) show that pancreatic β-cell function (%β), assessed by Homeostasis Model Assessment (HOMA) in patients allocated to diet or OHA decreased approximately 25% in 5 years[4]. In addition, a decline in endogenous insulin secretion over more than several decades of diabetes in patients including insulin-treated patients was observed in a cross-sectional study[5].

Determination of fasting serum C-peptide level and stimulated serum C-peptide level by intravenous glucagon is used widely to assess endogenous insulin secretory reserves[6–9], and the utility of the indices using C-peptide level in choosing insulin therapy has been shown[10]. The postprandial serum C-peptide level can easily be measured in clinical practice and has a good correlation with the serum C-peptide level after glucagon load[11]; it is often used as an index of endogenous insulin secretion, and can be used for both non-insulin-treated and insulin-treated patients[11–13]. Duration of diabetes and body mass index (BMI) are the major factors in serum fasting and glucagon-stimulated C-peptide levels[5,14], but the factors affecting postprandial serum C-peptide levels remain to be evaluated.

In the present study of Japanese patients with type 2 diabetes, to evaluate the clinical factors affecting postprandial serum C-peptide by cross-sectional study, 2-h postprandial C-peptide levels after breakfast were analyzed and compared with glucagon-stimulated C-peptide levels.

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SUBJECTS AND METHODS

Subjects

A total of 388 Japanese patients with type 2 diabetes who were admitted to Kyoto University Hospital between 1997 and 2002 for poor glycemic control were enrolled in the study. Patients with pancreatic or liver disease, taking diabetogenic medications, pregnant or with serum creatinine ≥1.3 mg/dL were excluded from the study. Type 2 diabetes mellitus was diagnosed based on the criteria of the American Diabetes Association (ADA)[15]. Patients with serum creatinine ≥1.3 mg/dL were excluded, as serum C-peptide immunoreactivity (CPR) is elevated by decreased renal function[16]. Of these patients, 115 were excluded as a result of incomplete clinical examinations and the remaining 273 patients, including patients without diabetic medication, oral hypoglycemic agent-treated patients and insulin-treated patients, were analyzed. The clinical profiles of the patients are shown in Table 1.

Methods

On the first day in hospital, medical history, physical examination and laboratory evaluation including glycosylated hemoglobin were carried out. HbA_{1c} was measured using high performance liquid chromatography (HA-8180; Arcray, Kyoto, Japan). The HbA_{1c} (%) value was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[17]. β-Cell function was evaluated within 1 week after an overnight fast by measuring fasting CPR (FCPR), CPR 6min after intravenous injection of 1 mg glucagon (CPR-6min)[6] and postprandial CPR. Serum CPR was measured by radioimmunoassay (Daiichi III; Daiichi Radioisotope Laboratories, Osaka, Japan). Postprandial CPR 2 h after breakfast (PPCPR) was determined. The meal at breakfast was prescribed as nutritional therapy according to the treatment guide for diabetes of the JDS[18], which included 516.6 ± 67.7 kcal (mean ± SD) energy consisting of 49% carbohydrate, 16% protein and 35% fat. In patients taking OHA, medication was stopped for measurement of CPR, but was maintained until 1 day before to prevent hyperglycemia during the test[5]. Plasma glucose was measured by the glucose oxidase method.

The study protocol was approved by the ethics committee of Kyoto University.

Table 1 | Clinical profiles of patients

No. patients	273
Male/female	158/115
Age (years)	61.2 ± 12.2
Years from diagnosis	9.6 ± 9.6
Systolic blood pressure (mmHg)	121.8 ± 12.9
Diastolic blood pressure (mmHg)	73.6 ± 9.6
BMI (kg/m^2)	23.9 ± 3.7
HbA_{1c} at admission (%)	9.7 ± 2.0
sCre (mg/dL)	0.69 ± 0.18
Glucagon load: FPG/PG-6min (mg/dL)	164.1 ± 47.9/180.6 ± 49.1
Glucagon load: FCPR/CPR-6min (ng/mL)	1.80 ± 0.97/3.83 ± 1.76
Meal load: FPG/PPPG (mg/dL)	167.0 ± 54.8/271.5 ± 83.5
Meal load: FCPR/PPCPR (ng/mL)	1.76 ± 0.94/4.87 ± 2.41

BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; FPG, fasting plasma glucose; OHA, oral hypoglycemic agents; PG-6min, plasma glucose 6 min after glucagon load; PPCPR, postprandial CPR; PPPG, postprandial plasma glucose; sCre, serum creatinine.

Statistical Analysis

Statistical analysis was carried out with the Stat View 5.0 system (SAS institute Inc., Cary, NC, USA). Data are presented as mean ± SD, unless otherwise noted. The relationship between the parametric clinical data and CPR values was investigated by Pearson's analysis. The relationship between the non-parametric clinical data and CPR values was investigated by Spearman's analysis. Clinical parameters among three groups were compared by analysis of variance (ANOVA). For comparison of two groups, Scheffé's test was carried out. *P*-values < 0.05 were considered statistically significant.

RESULTS

Simple correlation coefficients between FCPR, CPR-6min and PPCPR, and measures of variables (age, years from diagnosis, sex, BMI, systolic and diastolic blood pressure, HbA_{1c}, serum creatinine and plasma glucose [PG]) were calculated and are shown in Table 2. Years from diagnosis and BMI were significantly correlated with all three measures of CPR. PG and HbA_{1c} were significantly correlated with PPCPR ($P < 0.0001$, $r = -0.410$), but not with CPR-6min (Figure 1).

Stepwise multiple regression analysis was carried out using the independent variables in Table 2 to predict CPR as a dependent variable (Table 3). FCPR was independently predicted by years from diagnosis, BMI and serum creatinine, accounting for 22.4% of the variability of FCPR. CPR-6min was independently predicted by years from diagnosis and BMI, accounting for 17.9% of the variability of the dependent variables. PPCPR was independently predicted by years from diagnosis, BMI and HbA_{1c}, accounting for 31.5% of the variability of the dependent variables. Thus, HbA_{1c} is an important independent variable predicting PPCPR, but not FCPR or CPR-6min.

Because HbA_{1c} might be involved in decreased PPCPR, the clinical data among three groups of increased HbA_{1c} (≤8.5%, 8.6–10.3%, ≥10.4%) were compared, as shown in Table 4. Although there was no significant difference among these groups in FCPR and CPR-6min, PPCRP was significantly reduced with increasing levels of HbA_{1c}. CPR-6min was significantly correlated with PPCPR ($P < 0.0001$, $r = 0.564$, PPCPR = 0.774 × CPR-6min + 1.913; Figure 2a). PPCPR was correlated with CPR-6min in each tertile group of HbA_{1c} ($HbA_{1c} \leq 8.5$:

Table 2 | *P*-values and *r*-values of correlation between C-peptide immunoreactivity and measures of variables

	FCPR (ng/mL)	CPR-6min (ng/mL)	PPCPR (ng/mL)
Age (years)	0.4257 (ND)	0.0456 (−0.121)	0.3896 (ND)
Years from diagnosis	0.0024 (−0.182)	<0.0001 (−0.246)	0.0007 (−0.205)
Sex	0.0709 (ND)	0.1879 (ND)	0.8321 (ND)
BMI (kg/m^2)	<0.0001 (0.435)	<0.0001 (0.367)	<0.0001 (0.311)
Systolic blood pressure (mmHg)	0.5551 (ND)	0.9388 (ND)	0.0865 (ND)
Diastolic blood pressure (mmHg)	0.5739 (ND)	0.0327 (0.130)	0.0705 (ND)
HbA_{1c} (%)	0.0443 (−0.122)	0.1507 (ND)	<0.0001 (−0.410)
sCre (mg/dL)	0.0104 (0.155)	0.1641 (ND)	0.0140 (0.148)
FPG (mg/dL)	0.3764 (ND)	ND	ND
PG-6min (mg/dL)	ND	0.7333 (ND)	ND
PPPG (mg/dL)	ND	ND	<0.0001 (−0.285)

All correlations except correlations between sex and C-peptide immunoreactivity (CPR) were analyzed by Pearson's analysis. Correlations between sex and CPR were analyzed by Spearman's analysis. *P*-values are shown. In parenthesis, *r*-values are shown.
BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; FPG, fasting plasma glucose; ND, not determined; PG-6min, plasma glucose 6 min after glucagon load; PPCPR: postprandial CPR; PPPG: postprandial plasma glucose; sCre: serum creatinine.

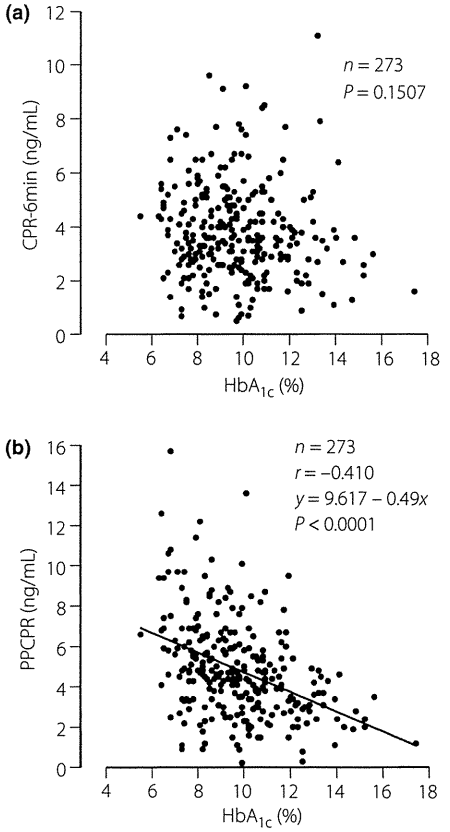

Figure 1 | The relationship between HbA_{1c} and (a) C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon (CPR-6min) and (b) 2-h postprandial C-peptide levels after breakfast (PPCPR).

$P < 0.0001$, $r = 0.595$, $y = 2.159 + 0.970x$, $n = 90$; $8.6\% \leq HbA_{1c} \leq 10.3\%$: $P < 0.0001$, $r = 0.674$, $y = 1.587 + 0.829x$, $n = 92$; $10.4\% \leq HbA_{1c}$: $P < 0.0001$, $r = 0.494$, $y = 2.091 + 0.482x$, $n = 91$). Because the higher HbA_{1c} group was distributed mainly below the regression line of total patients and the lower HbA_{1c} group above the line in the scattergram, and the increase in PPCPR per CPR-6min in the regression line of each tertile group was lower in the higher HbA_{1c} group, we examined the correlation between the ratio of PPCPR to CPR-6min (PPCPR/CPR-6min) and HbA_{1c}. PPCPR/CPR-6min was negatively correlated with HbA_{1c} ($P < 0.0001$, $r = -0.313$; Figure 2b).

DISCUSSION

In the present study, HbA_{1c} was negatively correlated with PPCPR, but not with FCPR or CPR-6min, which suggests that chronic elevation of the glucose level might impair endogenous insulin secretion after a meal load.

Although meal load is not equivalent to glucose load, as it contains nutrients other than carbohydrates that modulate glucose-induced insulin secretion, elevated glucose in plasma might play an important role in meal-stimulated insulin secretion. Indeed, the plasma glucose level after a meal load was increased considerably to more than 100 mg/dL in average. In contrast, the increment of glucose after glucagon load was only approximately 15 mg/dL, indicating a small contribution of glucose elevation to increased insulin secretion by glucagon loading.

Because HbA_{1c} was positively correlated with PPPG in the present study ($P < 0.0001$, $r = 0.570$), HbA_{1c} reflects postprandial glucose level. In simple correlation, both HbA_{1c} and PPPG were significantly correlated with PPCPR; whereas in stepwise regression analysis, HbA_{1c} was important to predict PPCPR, but PPPG was not. In addition, in simple correlation to PPCPR, the *r*-value for HbA_{1c} (0.410) was larger compared with that for PPPG (0.285; Table 2). These results show that PPCPR is more strongly affected by chronic elevation of glucose levels than by transient elevation of glucose levels.

Multiple regression analysis showed that years from diagnosis, BMI and HbA_{1c} were the major independent variables predicting PPCPR. This shows that years from diagnosis and BMI are common major factors predicting CPR. In contrast, HbA_{1c} was the major factor predicting PPCPR, but not FCPR or CPR-6min, and was negatively correlated with PPCPR. We hypothesized that CPR-6min reflects reserve capacity of endogenous insulin secretion independent of glycemic control and that PPCPR is predicted by a fundamental factor independent of glycemic control and by a variable factor dependent of glycemic control. CPR-6min predicted 31.8% of the variability of PPCPR as shown in Figure 2a. When a regression model using CPR-6min

Table 3 | Stepwise multiple regression analysis for predictors of C-peptide immunoreactivity

	F-value	Partial regression coefficient	Standard partial regression coefficient	R^2 (*R*)
FCPR (ng/mL)				
Years from diagnosis	9.4	−0.017	−0.170	0.224 (0.473)
BMI (kg/m^2)	55.2	0.108	0.406	
sCre (mg/dL)	7.3	0.823	0.149	
CPR-6min (ng/mL)				
Years from diagnosis	14.6	−0.039	−0.214	0.179 (0.423)
BMI (kg/m^2)	38.9	0.170	0.349	
PPCPR (ng/mL)				
Years from diagnosis	23.4	−0.063	−0.252	0.315 (0.561)
BMI (kg/m^2)	27.5	0.178	0.270	
HbA_{1c} (%)	68.7	−0.516	−0.431	

BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; PPCPR, postprandial CPR; sCre, serum creatinine.

Table 4 | Comparison of clinical characteristics and clinical profile among groups according to HbA_{1c} at admission

Groups (HbA_{1c} at admission)	I (≤8.5%)	II (8.6–10.3%)	III (≥10.4%)	*P*
No. patients	90	92	91	
HbA_{1c} (%)	7.6 ± 0.1	9.5 ± 0.1*	12.0 ± 0.1*†	<0.0001
Sex (male/female)	53/37	55/37	50/41	
Age (years)	64.2 ± 1.2	61.6 ± 1.3	57.6 ± 1.3*	0.0011
BMI (kg/m^2)	24.2 ± 0.3	24.1 ± 0.4	23.5 ± 0.4	0.3579
Years from diagnosis	11.7 ± 1.2	9.7 ± 0.8	7.4 ± 0.8*	0.0088
bSBP (mmHg)	122.9 ± 1.4	120.6 ± 1.3	121.3 ± 1.4	0.4746
DBP (mmHg)	72.9 ± 1.2	72.7 ± 1.0	75.3 ± 0.9	0.1302
sCre (mg/dL)	0.74 ± 0.02	0.70 ± 0.02	0.63 ± 0.02*†	<0.0001
FPG (mg/dL)	134.2 ± 3.7	163.4 ± 3.9*	195.0 ± 5.4*†	<0.0001
PG-6min (mg/dL)	152.4 ± 3.9	178.7 ± 4.0*	211.1 ± 5.6*†	<0.0001
PPPG (mg/dL)	223.3 ± 6.4	268.2 ± 7.7*	323.7 ± 8.9*†	<0.0001
FCPR (ng/mL)	1.92 ± 0.10	1.84 ± 0.10	1.66 ± 0.11	0.2004
CPR-6min (ng/mL)	3.85 ± 0.18	3.97 ± 0.19	3.66 ± 0.18	0.5467
PPCPR (ng/mL)	5.90 ± 0.29	4.88 ± 0.24*	3.86 ± 0.18*†	<0.0001

Data are presented as mean ± SE.
*$P < 0.01$ vs group I, †$P < 0.01$ vs group II.
BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; DBP, diastolic blood pressure; FCPR, fasting CPR; FPG, fasting plasma glucose; PG-6min, plasma glucose 6 min after glucagon load; PPCPR, postprandial CPR; PPPG, postprandial plasma glucose; SBP, systolic blood pressure; sCre, serum creatinine. FPG and FCPR are values when meal load was carried out.

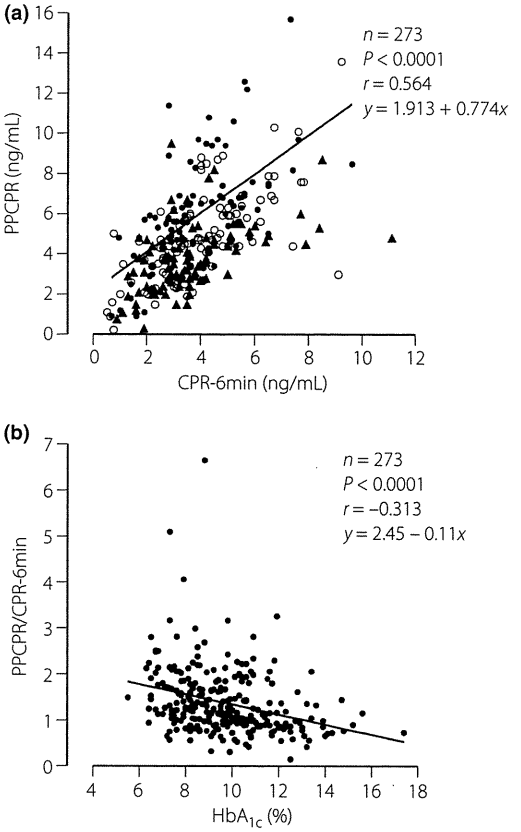

Figure 2 | Relationship between (a) C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon (CPR-6min) and 2-h postprandial C-peptide levels after breakfast (PPCPR) and (b) PPCPR/CPR-6min and HbA_{1c}. Black circles, $HbA_{1c} \leq 8.5\%$; white circles, $8.6\% \leq HbA_{1c} \leq 10.3\%$, black triangles, $10.4\% \leq HbA_{1c}$

and HbA_{1c} as independent variables to predict PPCPR as a dependent variable was used, CPR-6min and HbA_{1c} predicted 44.9% of the variability of PPCPR ($P < 0.0001$, $R = 0.670$, PPCPR = 6.286 + 0.730 × CPR-6min − 0.434 × HbA_{1c}). The addition of HbA_{1c} as an independent variable increased the prediction of the variability of PPCPR by 13.1%. In the present study, PPCPR/CPR-6min was used as a putative index of variability dependent of glycemic control and was found to be correlated with HbA_{1c} in the present study (Figure 2b). Furthermore, improvement of glycemic control by treatment ameliorates the CPR response after oral glucose load[19–21]. In addition, the CPR response after glucagon load is affected little by treatment to improve hyperglycemia and it is not correlated with the CPR response after oral glucose load before treatment, whereas it is well-correlated with improved CPR response after oral glucose load after treatment[21]. Reversible impairment of endogenous insulin response after glucose load is explained by glucose toxicity, in which chronic hyperglycemia deteriorates meal-induced and glucose-induced insulin secretion and insulin-sensitive glucose disposal[22]. Therefore, the chronic high glucose

level shown by high HbA_{1c} might impair endogenous insulin secretion after meal load, but has little effect on endogenous insulin secretion after glucagon load. The lack of influence of HbA_{1c} on CPR-6min might be helpful to evaluate reserve capacity of endogenous insulin secretion, even when glycemic control is poor enough to deteriorate postprandial insulin secretion. In contrast, PPCPR is affected by HbA_{1c} and might reflect the state of deteriorated insulin secretion by glucose toxicity that may be recovered by improved glycemic control.

In stepwise regression analysis, HbA_{1c} was not important to predict FCPR, but was important to predict PPCPR. In simple correlation, HbA_{1c} was significantly negatively correlated not only with PPCPR, but also with FCPR, whereas the *P*-value and *r*-value for FCPR were larger and smaller, respectively, compared with those for PPCPR (Table 2). Taken together, these findings suggest that glucose toxicity might deteriorate not only postprandial insulin secretion, but also fasting insulin secretion, whereas postprandial insulin secretion might be more vulnerable to glucose toxicity than to fasting insulin secretion.

The suppressive effect of glucose toxicity on insulin secretion *in vivo* might be attributable to impairment of β-cell responsiveness to glucose[22] and to impairment of incretin effect[23,24]. However, it is important to understand why glucagon-stimulated CPR is preserved despite severe impairment of glucose-stimulated CPR before treatment to improve hyperglycemia[21]. This remains largely unknown, but our hypothesis based on an *in vitro* study is that deteriorated intracellular glucose metabolism plays an important role in impaired glucose-induced insulin secretion[25] and that increased intracellular cyclic adenosine 3′,5′-monophosphate concentration derived from glucagon stimulation ameliorates impaired intracellular glucose metabolism to improve suppressed insulin secretion[26].

A recent study showed that indices using CPR correlate well with β-cell mass by analysis of β-cell areas of samples obtained during pancreatectomy and serum levels of CPR before operation[27]. Thus, PPCPR might reflect not only β-cell mass, but also reversible impairment of endogenous secretion as a result of chronic glucose elevation.

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Impact of endogenous and exogenous insulin on basal energy expenditure in patients with type 2 diabetes under standard treatment[1–3]

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ABSTRACT

Background: Factors that affect resting energy expenditure or basal energy expenditure (BEE) in patients with type 2 diabetes under standard treatment have not been evaluated in detail.

Objective: We determined the clinical factors that affected BEE in addition to body composition in patients with type 2 diabetes under standard treatment.

Design: BEE was measured by using indirect calorimetry under a strict basal condition in 58 Japanese patients with type 2 diabetes after >7 d as inpatients under management of diabetes with medical nutrition therapy and medications. Insulin secretion was measured with a glucagon test. Stepwise regression was applied to explore determinants of BEE.

Results: In the stepwise estimation, insulin secretion ($P = 0.015$), insulin therapy ($P = 0.012$), and pulse rate ($P = 0.011$) were selected in addition to fat-free mass (FFM) ($P < 0.001$) and fat mass ($P = 0.006$) as significant independent determinants of BEE. Standardized partial regression coefficients of the additional 3 factors were −0.16, −0.15, and 0.15, respectively, whereas those for FFM and fat mass were 0.82 and 0.19, respectively. The additional 3 factors explained another 3.9% of the variability of BEE, and the adjusted coefficient of determination was 83.4%. Age, sex, other medications, and parameters of glycemic control were not significant determinants beyond the combined contribution of body composition, endogenous and exogenous insulin, and pulse rate.

Conclusion: Endogenous insulin secretion and exogenous insulin administered in treatment have significant independent effects in the lowering of BEE in patients with diabetes under standard management with medical nutrition therapy and medications. *Am J Clin Nutr* 2011;94:1513–8.

INTRODUCTION

MNT[4] is the basis and starting point of treatment of all patients with diabetes, and the failure of MNT alone may predict the inability to attain optimal glycemic control. In addition, in obese patients with diabetes, MNT aims at weight control to improve insulin resistance and avoid obesity-related health problems. Therefore, estimation of the daily energy expenditure of patients is necessary for individualized diabetic meal plans. For example, MNT for obese patients provides a diet of 500–1000 kcal less energy than the estimated energy expenditure (1). REE or BEE is defined as the minimal amount of energy expended to maintain metabolic activities of cells, tissues, and organs and represents a large component of daily energy expenditure. REE or BEE is used to estimate total energy expenditure as a multiple of REE or BEE according to daily activity.

In healthy subjects, 65–90% of the interindividual variation in REE is explained by FFM or high-metabolic-rate organ mass (2, 3). In patients with diabetes, FFM is also assumed to be a main factor that affects REE and BEE. The influence of the diabetic condition on energy expenditure has also been examined. After adjustment for FFM, REE and BEE in patients with diabetes were shown to be greater than those in healthy control (4, 5). In contrast, no difference was reported in FFM-adjusted REE between mildly hyperglycemic patients and control subjects (6). In addition, a treatment-induced reduction of REE has been reported in type 1 and type 2 diabetes (7, 8). However, the factors that affect REE or BEE in patients with diabetes under standard treatment, which are the more important in clinical practice, have not been precisely evaluated.

In most studies that evaluated energy expenditure, REE has been measured rather than BEE. However, resting conditions are defined less rigorously than are basal conditions, and REE can include components that involve physical or psychological stress and variations in ambient and body temperature (9–11). BEE is measured early in the morning before the subject has engaged in any physical activity and ≥10 h after ingestion of any food, drink, or nicotine and remains remarkably constant on a daily basis (9, 11). We applied the measurement of BEE under such

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[4] Abbreviations used: A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; EGO, endogenous glucose output; FFM, fat-free mass; FPG, fasting plasma glucose; Hb A_{1c}, glycated hemoglobin; MNT, medical nutrition therapy; PG, plasma glucose; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; REE, resting energy expenditure.

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strict basal conditions to minimize the interindividual variation derived from methodologic or external unknown factors.

In the current study, clinical factors that might affect BEE in patients with diabetes under standard treatment were evaluated.

SUBJECTS AND METHODS

Subjects

Japanese patients with type 2 diabetes who were admitted to the Department of Diabetes and Clinical Nutrition, Kyoto University Hospital, Kyoto, Japan, for diabetes self-management education during the period of December 2007 through September 2009 were recruited. The study protocol was approved by the ethics committee of Kyoto University. Written informed consent was obtained from all participants.

During admission, which averaged ~18 d, participants took MNT with or without medications, including oral hypoglycemic agents and insulin, according to the treatment guide for diabetes of the Japan Diabetes Society (12). The physical activity of subjects was not restricted, but participants did not engage in intensive exercise. Participants were screened by a medical history, physical examination, and laboratory examination to ensure the absence of hepatic, renal (including macroalbuminurea), pulmonary, thyroid, and cardiac dysfunction, inflammation, and malignant tumors. Participants who took steroids or β-blockers and subjects who had physical disabilities were excluded.

Energy expenditure

BEE was measured in the morning >7 d after admission under glycemic control with MNT (29.0 ± 2.6 kcal/kg of standard body weight/d that consisted of 52% carbohydrate, 20% protein, and 28% fat in energy-intake percentages) and with without prescribed medications. Standard body weight (in kg) was calculated by multiplying 22 (kg/m^2) by square of height (in m). Premenopausal women were studied during their follicular phase within 7 d after the last day of menstruation.

Whole-body oxygen consumption and carbon dioxide production were measured for >10 min with indirect calorimetry (AE300S; Minato Medical Science) by one investigator at the bedside of each participant under the strict condition of the methods described previously (9, 10, 13). Briefly, afebrile patients in a postabsorptive state after an overnight fast (14 h) with <10 mmol capillary PG/L before the measurement remained in a supine position after waking on the bed in the ward without smoking or taking caffeine, and measurements were performed at room temperature between 22°C and 27°C. After discarding the initial 5 min of data, we adopted 5 consecutive minutes of data from the rest in accord with the steady state definition (10) during which the CV for oxygen consumption and carbon dioxide production was achieved at ≤10% and applied them to the Weir formula together with 24-h urinary urea nitrogen (14).

Body composition

Height was measured on the day of admission, and body weight was measured immediately after measurement of BEE. FFM and fat mass were measured with a dual energy X-ray absorptiometry scanner (Discovery; Hologic) within 3 d before or after the measurement of BEE.

Clinical and metabolic factors

The duration of diabetes was determined from medical records, medical histories, and previous clinical data according to the criteria for the diagnosis of diabetes proposed by the American Diabetes Association (15). Hb A_{1c} was measured by using HPLC (ADAMS A1C HA8180; Arcray) and expressed as a National Glycohemoglobin Standardization Program equivalent value (A_{1c}; percentage) calculated by the formula

$$\text{Percentage of } A_{1c} = \text{percentage of Hb } A_{1c} \text{ (Japan Diabetes Society)} + 0.4\% \quad (1)$$

which considers the relational expression of Hb A_{1c} (Japan Diabetes Society) (percentage) measured by using the previous Japanese standard substance and measurement methods and Hb A_{1c} (National Glycohemoglobin Standardization Program) (16). Thyroid stimulating hormone was measured by using electrochemiluminescence immunoassay (Elecsys Anti-Tg; Roche Diagnostics GmbH) to confirm the absence of thyroid dysfunction. Capillary glucose before each meal was measured with a glucose meter (One Touch Ultra; Johnson & Johnson) and expressed as capillary PG. As a variable of glycemic control, the mean preprandial PG for 3 consecutive days before the measurement of BEE and FPG just before the measurement of BEE was used. The mean pulse rate was calculated from the records of checkups by nurses on 3 consecutive days including the day of BEE measurement.

Insulin secretion

β cell function was evaluated after an overnight fast by measuring CPR6′ (17) because the test is valid in patients who take insulin therapy. The serum C-peptide immunoreactivity was measured by using an immunoenzymometric assay (ST AIA-PACK C-peptide; Toso). On the morning of the glucagon test, participants took their medication after the test.

Statistical analysis

Descriptive data were expressed as means ± SDs. Information of medications, including insulin, sulfonylurea, and metformin, was coded as use = 1 and nonuse = 0. Sex was coded as male = 1 and female = 0. The Mann-Whitney U and Fisher's exact tests were performed to identify differences in the characteristics of men and women. The interrelation between BEE and clinical factors was investigated by means of Kendall's rank correlation coefficients. Clinical factors include body composition (FFM and fat mass), FPG, mean preprandial PG, A_{1c}, insulin secretion, mean pulse rate, dietary energy, and medications (ie, insulin, sulfonylurea, and metformin). A multiple linear regression analysis was performed to evaluate the contribution of each determinant to BEE. Variables were selected by stepwise estimation from age, sex, and the clinical factors previously described. When P was <0.05 and ≥0.15, the variable was added and removed, respectively. Data were analyzed by use of Stata 11.0 software (StataCorp). Statistical significance was set at $P < 0.05$ (2-tailed).

TABLE 1
Characteristics of participants[1]

	All	Men	Women
Patients (*n*)	58	35	23
Age (y)[2]	60.2 ± 9.2[3]	57.6 ± 9.7	64.0 ± 7.1
Body weight (kg)[2]	63.7 ± 15.6	68.3 ± 16.5	56.8 ± 11.2
BMI (kg/m^2)	24.2 ± 5.0	24.1 ± 5.6	24.4 ± 4.1
FFM (kg)[2]	48.4 ± 11.1	54.1 ± 9.4	39.7 ± 7.1
BEE (kcal/d)[2]	1294 ± 227	1397 ± 217	1136 ± 135
CPR6′ (ng/mL)	3.4 ± 1.8	3.4 ± 2.0	3.3 ± 1.6
FPG (mg/dL)	114.5 ± 25.2	113.7 ± 25.1	115.6 ± 25.9
PPPG (mg/dL)	143.9 ± 35.5	147.5 ± 37.6	138.5 ± 32.0
A_{1c} (%)	10.5 ± 2.5	10.3 ± 2.4	10.9 ± 2.7
Pulse rate (beats/min)	72.7 ± 10.1	75.0 ± 10.8	69.2 ± 7.8
Duration of diabetes (y)	8.4 ± 7.0	10.0 ± 7.9	5.9 ± 4.4
Treatment			
Diet (kcal · SBW^{-1} · d^{-1})	28.8 ± 2.4	28.7 ± 2.0	29.0 ± 3.0
Medications (*n*)			
Insulin only	24	17	7
Insulin + metformin	10	4	6
Insulin + sulfonylurea	3	2	1
Insulin + sulfonylurea + metformin	1	0	1
Sulfonylurea only	8	5	3
Sulfonylurea + metformin	5	2	3
Metformin only	4	3	1
None	3	2	1

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; FFM, fat-free mass; FPG, fasting plasma glucose just before the measurement of BEE; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; SBW, standard body weight.
[2] Mann-Whitney *U* test revealed significant ($P < 0.05$) differences between men and women.
[3] Mean ± SD (all such data).

RESULTS

Participant characteristics and results of measurement are shown in **Table 1**. BMI (in kg/m^2), CPR6′, FPG, PPPG, A_{1c}, pulse rate, duration of diabetes, and treatment did not differ significantly between men and women, whereas body weight, FFM, and BEE were significantly higher in men, and age was higher in women.

Significant positive correlations were observed between BEE and FFM ($r = 0.72$) and dietary energy ($r = 0.33$), but dietary energy also had a significant positive correlation with FFM ($r = 0.37$) and a significant negative correlation with fat mass ($r = -0.38$) (**Table 2**). Fat mass had a significant negative correlation with mean preprandial PG ($r = -0.30$) and a significant positive correlation with insulin secretion (CPR6′) ($r = 0.43$)

TABLE 2
Correlations between BEE and possible determinants of BEE[1]

	BEE	FFM	FM	FPG	PPPG	A_{1c}	CPR6′	PR	Diet	Insulin[2]	Sulfonylurea[2]
BEE	1.00	—	—	—	—	—	—	—	—	—	—
FFM	0.72*	1.00	—	—	—	—	—	—	—	—	—
FM	0.18†	0.16	1.00	—	—	—	—	—	—	—	—
FPG	−0.05	−0.08	−0.13	1.00	—	—	—	—	—	—	—
PPPG	−0.08	−0.08	−0.30*	0.42*	1.00	—	—	—	—	—	—
A_{1c}	−0.07	−0.03	−0.14	0.16	0.18†	1.00	—	—	—	—	—
CPR6′	0.19†	0.19†	0.43*	0.03	−0.23†	−0.09	1.00	—	—	—	—
PR	0.21†	0.14	0.05	0.07	0.08	−0.06	0.07	1.00	—	—	—
Diet	0.33*	0.37*	−0.38*	0.07	0.20†	0.06	−0.14	0.09	1.00	—	—
Insulin[2]	−0.12	−0.06	−0.18	0.28†	0.37*	0.27†	−0.24†	0.13	0.00	1.00	—
Sulfonylurea[2]	0.01	−0.00	0.12	−0.36‡	−0.28†	−0.11	0.07	−0.15	−0.06	−0.57*	1.00
Metformin[2]	0.18	0.15	0.40*	−0.13	−0.38*	−0.01	0.29‡	0.05	−0.22	−0.16	0.01

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure (kcal/d); CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection (ng/mL); Diet, energy of diet (kcal/d); FFM, fat-free mass (kg); FM, fat mass (kg); FPG, fasting plasma glucose just before the measurement of BEE (mg/dL); PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE (mg/dL); PR, pulse rate (beats/min). *†,‡Kendall's correlation coefficients ($n = 58$): *$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$.
[2] Use = 1; nonuse = 0.

TABLE 3
Regression models for BEE and body composition and other clinical factors[1]

	Coef (95% CI)	SE	Std coef	*P*	Adjusted R^2
Model 1					
BEE (FFM + FM)					0.795
Intercept	400.0 (277.1, 523.1)	61.4	—	<0.001	
FFM	17.0 (14.3, 19.7)	1.3	0.83	<0.001	
FM	4.3 (0.3, 8.4)	2.0	0.14	0.038	
Model 2[2]					
BEE (FFM + FM + CPR6′ + INS + PR)					0.834
Intercept	256.2 (59.3, 453.0)	98.1	—	0.012	
FFM	16.9 (14.3, 19.4)	1.3	0.82	<0.001	
FM	5.8 (1.8, 9.8)	2.0	0.19	0.006	
CPR6′	−19.9 (−35.6, −4.1)	7.9	−0.16	0.015	
Insulin[2]	−69.9 (−124.0, −15.7)	27.0	−0.15	0.012	
PR	3.3 (0.8, 5.9)	1.3	0.15	0.011	

[1] Model 1 predicted BEE only from body composition by linear regression analysis (n = 58). The final regression model (model 2) used independent variables selected from FFM, FM, age, sex, CPR6′, fasting plasma glucose, mean preprandial plasma glucose, A_{1c}, dietary energy, use of insulin, use of metformin, use of sulfonylurea, and pulse rate by stepwise estimation (n = 58). A_{1c}, glycohemoglobin (%); BEE, basal energy expenditure (kcal/d); Coef, partial regression coefficient; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection (ng/mL); FFM, fat-free mass (kg) measured by using dual energy X-ray absorptiometry; FM, fat mass (kg) measured by using dual energy X-ray absorptiometry (kg); INS, insulin; PR, pulse rate (beats/min); Std coef, standardized coefficient.

[2] Use = 1; nonuse = 0.

and use of metformin (r = 0.40). FPG had a negative correlation with the use of sulfonylurea (r = −0.36). Mean preprandial PG had a positive correlation with the use of insulin (r = 0.37) and a negative correlation with the use of metformin (r = −0.38). Insulin secretion (CPR6′) had a negative correlation with the use of insulin (r = −0.24) and a positive correlation with the use of metformin (r = 0.29).

Because clinical factors were naturally correlated with each other, possible spurious correlation and suppressor variables were investigated by means of multiple regression analysis. The model that predicted BEE from body composition alone is shown in **Table 3** (model 1). Another model included selected variables by stepwise estimation (model 2). Insulin secretion (CPR6′; P = 0.015), use of insulin (P = 0.012) and pulse rate (P = 0.011) were significant determinants of BEE in addition to FFM (P < 0.001) and fat mass (P = 0.006). These additional factors explained another 3.9% of the variability of BEE, and the adjusted coefficient of determination was 83.4%. Standardized coefficients of FFM, fat mass, insulin secretion (CPR6′), use of insulin, and pulse rate in model 2 were 0.82, 0.19, −0.16, −0.15, and 0.15, respectively. Variance inflation factors of variables in model 2 were all <1.5. Other plausible determinants (ie, FPG, mean preprandial PG, A_{1c}, dietary energy, use of sulfonylurea, use of metformin, age, and sex) were not selected as significant contributors by stepwise estimation. The effect of insulin secretion (CPR6′) in BEE adjusted for FFM, fat mass, use of insulin, and pulse rate is illustrated in **Figure 1**.

DISCUSSION

In the current study, endogenous insulin secretion and exogenous insulin administered in treatment were both significant independent variables that predicted BEE in Japanese patients with type 2 diabetes under MNT and medications. In addition, both factors were negatively correlated with BEE. The current results suggested that the effect of insulin, regardless of the endogenous or exogenous source, had a significant negative impact on energy expenditure in patients with fair glycemic control under medical care.

In the current study, clinical factors regarding the effects of insulin predicted BEE, whereas factors regarding glycemic control did not predict BEE. The cause of these differing results is not clear, but the present results indicated that the effects of insulin affected BEE independently of glycemia. In a previous report, EGO was shown to be a significant predictor of elevated REE of patients with diabetes after adjustment for body composition, but the glucose concentration was not (18). Although FPG was correlated with EGO, it accounted for only 21% of its variability (19). Gluconeogenesis is an energy-consuming metabolic pathway and is thought to be a major source of increased EGO in diabetes (20–22). Because hepatic insulin deficiency plays an important role in increased gluconeogenesis in diabetes

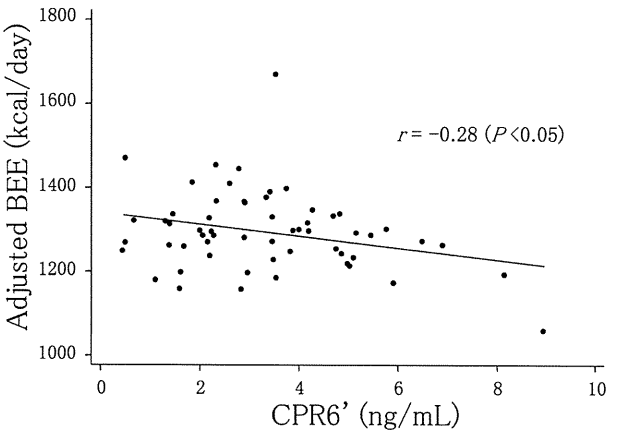

FIGURE 1. Relation between CPR6′ and BEE after adjustment for fat-free mass, fat mass, pulse rate, and insulin use (n = 58). BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection.

(19), insulin, both endogenous and exogenous, may suppress BEE by reducing gluconeogenesis.

Protein turnover is also involved in energy expenditure. Protein turnover is often elevated in diabetes and has been shown to be correlated with REE in type 2 diabetes (23, 24). Because the treatment-induced reduction of nitrogen flux, which is an index of protein turnover, has been shown to be correlated with the C-peptide response to an oral glucose challenge (8), protein turnover may well play a role in the alteration of energy expenditure by insulin. In addition, impairment of insulin release is often accompanied by hyperglucagonemia in fasting and postprandial states in type 2 diabetes (25). Hyperglucagonemia is responsible for increased leucine oxidation and REE by insulin deprivation in type 1 diabetes (26). In addition, thermogenesis, especially via β-3 adrenergic receptors, has another impact on energy expenditure (20, 27). Considered together, these findings suggested that glucagon and catecholamine may also affect BEE in type 2 diabetes. However, we did not measure glucagon and catecholamine, which was a limitation of the current study.

Contrary to our results, the fasting insulin concentration has been reported to be positively correlated with energy expenditure (18). In the report (18), the positive correlation between insulin and energy expenditure was speculated to derive from the activation of the sympathetic nervous system by insulin (28). We showed that CPR6′ and insulin use were negatively correlated with BEE independently of the pulse rate, which is an indicator of the sympathetic nerve system activity, and was positively correlated with BEE independently of CPR6′ and insulin use. These findings suggested that the effect of insulin on BEE may be independent of the sympathetic nerve system activity at least in patients under treatment.

The difference in insulin sensitivity between lean Japanese subjects and obese American subjects may account for the difference in association of insulin with BEE. In general, a greater attribution of impaired insulin secretion and a lesser contribution of insulin resistance to glucose intolerance were shown in Japanese patients with type 2 diabetes, whose average BMI is ~25 but which is >30 in European and American populations (29). This difference may be attributable, in part, to the difference in BMI; in the Swedish population, the average CPR6′ is lower (~2 ng/mL) in lean subjects with BMI of ~25 than it is in obese subjects (~8 ng/mL) with BMI of ~30. Thus, our findings are in accord with those in Swedish lean subjects (30). Our previous finding of a positive correlation between BMI and CPR6′ also supported this attribution of difference in insulin secretion to difference in BMI (31).

Glycemia was shown to not be an independent determinant of BEE in this study. Although elevated BEE or REE after adjustment for body composition in patients with diabetes compared with in healthy subjects has been reported, the glucose concentration was not described (4, 5). Another study reported a significantly greater REE in patients with high glucose values (>10 mmol/L) compared with in patients with low glucose values (<10 mmol/L) (32). In addition, no increase in BEE was shown in patients with glycemic concentrations of ~9.5 mmol/L (6). Blood glucose concentrations of patients in the current study were improved by treatment, and FPG concentrations and mean preprandial PG concentrations were ~115 mg/dL (6.4 mmol/L) and 144 mg/dL (8.0 mmol/L) respectively. Therefore, the finding of no association between BEE and PG concentrations in the current study was not discrepant with previous reports and was in accord with the finding that REE did not significantly correlate with fasting glucose in type 2 diabetes in which the fasting glucose concentration was ~7.4 mmol/L (18). Together, these data suggested that glycemia is not an important factor in the prediction of BEE in patients with diabetes under treatment.

Dietary energy, treatment with sulfonylurea or metformin, age, and sex were not significant variables that predicted BEE. Sex itself was not a significant determinant but affected coefficients of FFM and fat mass when added to model 2. Researchers have noted a contribution of sex to BEE in addition to FFM and fat mass, although information of menstruation at the measurement of BEE was absent (4, 5). In the current study, BEE was measured during the follicular phase in accordance with a strict protocol for premenopausal women to avoid the influence of progesterone, which was supposed to increase energy expenditure. Another possibility is that our sample size was not large enough to show the contribution of sex to BEE. Last, with consideration of our previous finding that CPR6′ was independently influenced by BMI and the duration of diabetes in type 2 diabetes (31), we performed a regression analysis by adding these 2 putative covariates to model 2, which showed the insignificance of these determinants.

In conclusion, the current study shows that endogenous and exogenous insulin both have a significant impact on lowering BEE in patients with type 2 diabetes under standard treatment with MNT and medications. Longitudinal studies are required to elucidate the impact of the increment of C-peptide concentrations by insulinotropic agents and the administration of insulin on energy expenditure and gluconeogenesis during fair glycemic control.

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The authors' responsibilities were as follows—KI, SF, MG, and TK: designed the research; KI, CY, AH, and KS: conducted the research; KI, MG, and SF: analyzed data; KI and SF: wrote the manuscript; NI: supervised the research; SF: had primary responsibility for the final content of the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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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:

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

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

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

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

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

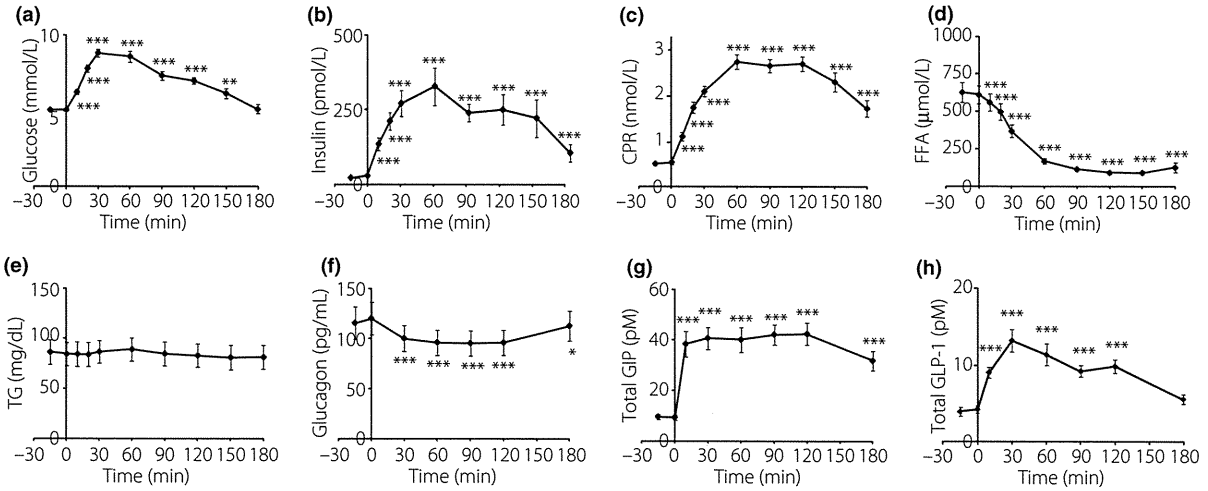

Figure 1 | Concentrations of (a) plasma glucose, (b) serum immunoreactive insulin, (c) serum C-peptide reactivity (CPR), (d) serum free fatty acid (FFA), (e) serum triglyceride (TG), (f) glucagon, (g) total gastric inhibitory polypeptide (GIP) and (h) total glucagon-like peptide-1 (GLP-1) during oral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

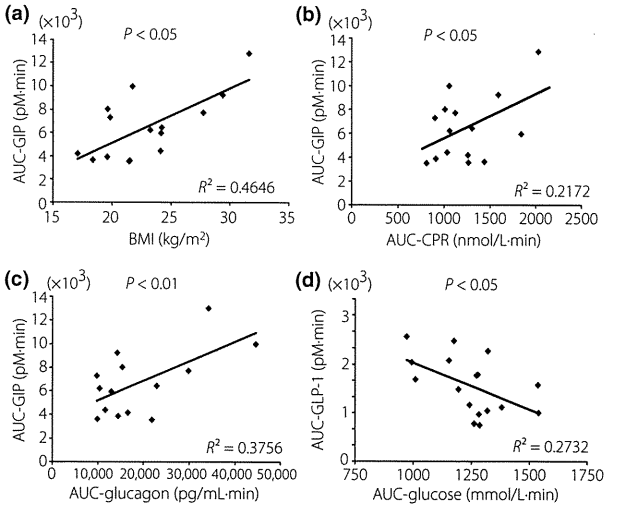

Figure 2 | Simple regression analysis of gastric inhibitory polypeptide secretion (AUC-GIP) and (a) body mass index (BMI), (b) AUC of serum C-peptide reactivity (CPR) and (c) glucagon. (d) Simple regression analysis of glucagon-like peptide-1 secretion (AUC-GLP-1) and AUC of plasma glucose (PG).

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

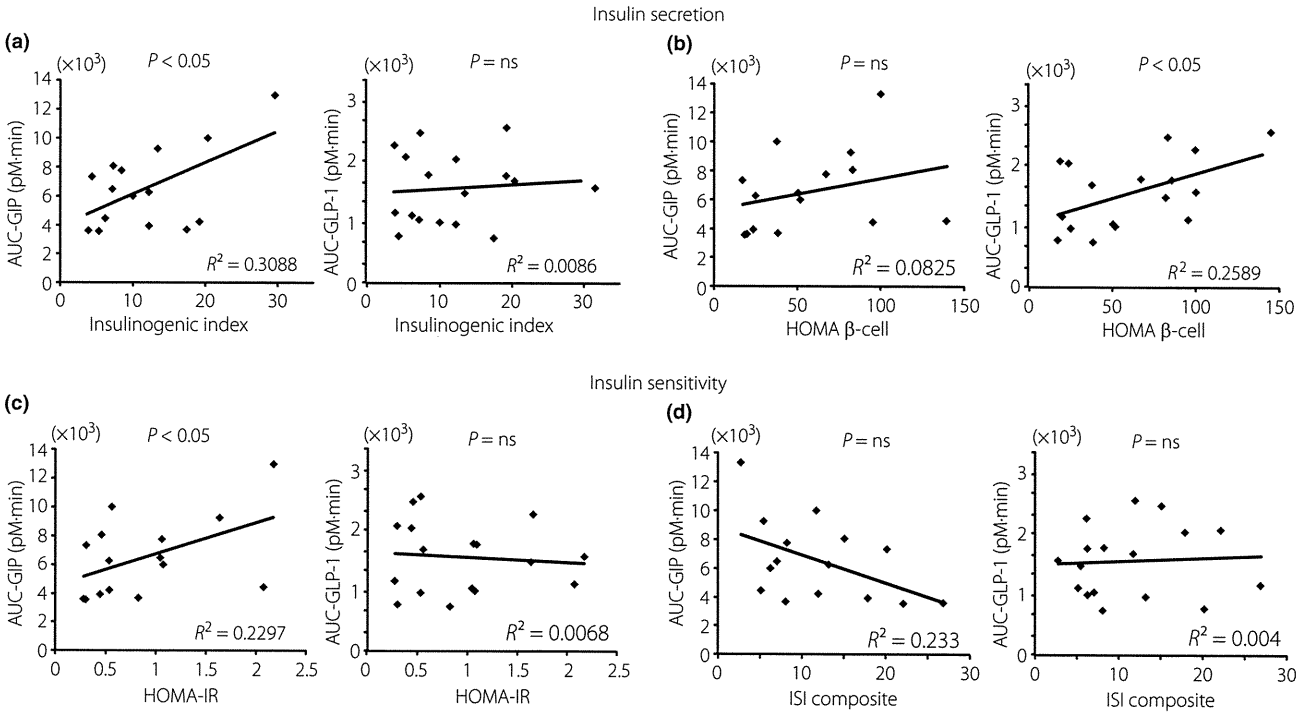

Figure 3 | Relationship between gastric inhibitory polypeptide secretion (AUC-GIP) and glucagon-like peptide-1 secretion (AUC-GLP-1) and the indices of insulin secretion and insulin sensitivity. (a) Insulinogenic index, (b) homeostasis model assessment (HOMA) β-cell function, (c) homeostasis model assessment 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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