

Obesity and Diabetes Mellitus

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Received date: February 09, 2014, Accepted date: March 28, 2014, Published date: Apr 06, 2014

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Abstract

Backgrounds: Generally, a prevalence of obesity increasing constantly represents one of major health care and social problems. Many researchers indicate that obesity has a risk factor for type 2 diabetes mellitus (T2DM), but some persons believe that obesity may occur T2DM. GLP-1 and GIP as incretin hormone are secreted in response to ingestion of nutrients. In the circulation, they are rapidly inactivated by dipeptidyl peptidase-4. We report interesting findings on secretion of incretin after test meal (TM) in Japanese patients with type 1 diabetes mellitus (T1 DM) and T2DM associated with or without obesity.

Materials and Methods: In Japan, ≥ 25 kg/m² in BMI are defined as obesity. After overnight fast, subjects were ingested of TM (550 kcal) comprised of 60% carbohydrate, 23% fat and 17% protein. Based on GLP-1, patients with T1DM (n=10) were treated with multiple daily injections of insulin (MDI) or CSII. Non-obese (n=23) and obese (n=24) patients with T2DM with micro- and macroangiopathy were treated with oral drugs for various disease. Based on GIP, patients with T1DM (n=15) and T2DM (n=29) were treated with MDI or CSII for T1DM and oral drugs for T2DM, respectively.

Results: Basal and postprandial levels of plasma active GLP-1 (p-GLP-1) after TM in Japanese patient with T1DM and T2DM are similar to those with control, but basal and postprandial ratio of p-GLP-1/glucose are low compared with controls. AUCs of plasma GIP at early-phase were significant negatively and positively related to BMI in patients with T1DM and T2DM, respectively.

Conclusions: Japanese patients with T2DM regards of obesity may have a low secretion of GLP-1, which may be due to genetic factors. However, there is no T2DM in obese persons without low secretion of GLP-1. Therefore, risk factors for DM are important to diagnose T2DM.

Keywords: GLP-1; GIP; BMI; Non-obesity; Obesity; Type 2 DM; Type 1 DM

Introduction

Generally, a prevalence of obesity is constantly increasing, and it represents one of the major health care and social problems nowadays. Obesity is a risk factor for several clinical and metabolic problems. However, there is a huge individual variability in the risk for metabolic and clinical morbidity associated with obesity [1,2]. This has led to description in the medical literature of groups with obese subjects, in spite of having a high body mass index (BMI), being relatively resistant to development of clinical and metabolic abnormalities. These subjects have been referred as “Metabolically Healthy but Obese” (MHO) [3-6]. A systematic review of 14 recently published studies [4,5] found that the prevalence of MHO phenotype ranged widely from 6 to 44% [6].

In this chapter, we mentioned the relation of obesity and diabetes mellitus (DM) via our research of incretin secretion. Today, a definition of obesity based on BMI is different between the Japan, and the U.S.A. and the Europe [7]. In the Japan, ≥ 25.0 kg/m² in BMI are defined as obesity, whereas in the U.S.A. and the Europe, ≥ 30.0 kg/m²

in BMI are defined as obesity [7]. That is, a degree of obesity is different from a definition in countries. Further, DM is a group of disease associated with various metabolic disorders, of which main future is chronic hyperglycemia owing to insufficient insulin action, and is mainly divided into two types, type 1 (T1DM) and type 2 (T2DM) [8-11]. Generally, T1DM is based on the destruction of pancreatic β -cells, usually leading to absolute insulin deficiency, whereas T2DM is based on the ranging from predominantly insulin secretory defect to predominantly insulin resistance with varying degrees of insulin secretory defect [8-11]. Obese patients with T2DM have a strong insulin-resistance compared with non-obese patients with T2DM [8-15]. Accordingly, many researchers indicate that obesity has a risk factor for T2DM [8-11], but many peoples may believe that obesity occurred in T2DM. Is it true? Our works threw a doubt on direct questions at the problems and answered the question.

Incretin, principally two hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), regulates islet hormone secretion, glucose concentration, lipid metabolism, gut motility, appetite, body weight, and immune function, which provides a scientific basis for using incretin-based therapies in treatment of T2DM [12,13]. GLP-1 and GIP are secreted

in response to the ingestion of nutrients. In the circulation, they are rapidly inactivated by dipeptidyl peptidase-4 (DPP-4), which cleaves off two N terminal amino acids [2]. Recently, these hormones have attracted much interest in patients with DM [12,13].

Vilsbøll et al. [14] reported that a low response in postprandial plasma immunoreactive active GLP-1 (p-GLP-1) to ingestion of a breakfast test meal (TM) (560 kcal) was seen in insulin-naïve European patients with T2DM [14], whereas normal responses in postprandial p-GLP-1 and plasma GIP (p-GIP) were observed in European patients with T1DM [14]. Meanwhile, responses of postprandial p-GLP-1 and p-GIP levels following ingestion of TM (480 kcal) in non-obese or obese Japanese patients with T2DM were similar to those in controls [15,16].

The difference between by Vilsbøll et al. [14] and by Lee et al. [15] and Kozawa et al. [16] may be in part due to the difference of degree of BMI in subjects participated. Therefore, we re-examined effects of TM based on the method of Vilsbøll et al. [14] on basal and postprandial levels of incretin in non-naïve Japanese patients with T1DM or T2DM associated various degrees of BMI and in controls with normal glucose tolerance (NGT) [17-20].

Here, we report interesting findings on studies of non-obese and obese persons through our research of incretin secretion in non-naïve Japanese patients with DM [17-20].

Materials and Methods

Subjects

The selection criteria were non-obese or obese Japanese patients with DM, who visited our clinic regularly. The exclusion criteria were pregnant women, and persons with gastrointestinal diseases, or impaired liver function.

First, based on GLP-1 secretion, Japanese patients with T1DM (6 acute, 2 fulminant, and 2 slowly progressive) (n=10), who visited our clinics regularly and control subjects with NGT for a 75 g oral glucose tolerance test (75g-OGTT) (n=15) were studied [17]. Non-obesity and obesity were based on BMI by criteria of the Japan Society for the Study of Obesity [7]. Diabetic patients were diagnosed using the World Health Organization (WHO) criteria [8], which nowadays require more than 6.5% of glycosylated hemoglobin A1c (HbA1c) as NGSP (National Glycohemoglobin Standardization Program) by the Japan Diabetes Society (JDS) [10-11]. Patients with T1DM associated with hypoglycemia were excluded from this study, because that p-GLP-1 level in patents may be influenced owing to hypoglycemia [21]. Demographic characteristics of the participants are presented in Table 1. Patients with T1DM and control were matched by sex, age, and BMI. Chronic complications were defined as micro- and macrovascular disturbances. The occurrences of retinopathy (RP), nephropathy (RNP), and neuropathy (NP) as microvascular complications were examined by ophthalmologist (24), by means of albuminuria and by Achilles' tendon reflex, respectively. The macrovascular complications were defined by past medical history and the occurrences of inactive coronary heart disease (CHD) or inactive cerebral vascular disturbance (CVD) were assigned (Table 1). Although patients had a mean of 19 years duration from discovering the disease (Table 1), there had no such complications. As they had <0.5 ng/mL of fasting serum immunoreactive C-peptide (s-CPR) concentrations, they were treated with multiple daily injections of insulin (MDI) (four injections per day; 12-59 U/day) or CSII (33-42 U/

day) using insulin analogues (Table 1). One patient was treated with an angiotensin receptor blocker and a calcium antagonist for hypertension. Control was recruited from persons with NGT [17]. None of the participants had a history of gastrointestinal diseases, anemia, or impaired liver function, and none were receiving any other medications.

Variables	Type 1	Control	P value
Number	10	15	
Gender (female/male)	5/5	10/5	0.4420
Age (yr)	51 ± 5	49 ± 2	0.7340
BMI (kg/m ²)	22.3 ± 0.7	21.1 ± 0.4	0.1790
HbA1c (%) (NGSP)	7.3 ± 0.2	5.3 ± 0.04	<0.0001
Fasting plasma glucose (mg/dL)	164 ± 20	91 ± 1.7	<0.0001
Serum immunoreactive insulin (μU/mL)	4.9 ± 0.7	3.9 ± 0.7	0.3140
Serum immunoreactive C-peptide (ng/mL)	0.2 ± 0.01	1.3 ± 0.1	<0.0001
SUIT	0.4 ± 0.1	3.6 ± 0.3	0.0001
Plasma immunoreactive active GLP-1 (pmol/L)	1.7 ± 0.3	2.8 ± 0.6	0.1300
Ratio of plasma immunoreactive active GLP-1/ fasting plasma glucose (x10 ⁶)	0.22 ± 0.05	0.56 ± 0.11	<0.0001
Duration of therapy (year)	19.0 ± 3.0		
Chronic complications			
Microangiopathy	0		
Macroangiopathy	0		
Therapy			
Diabetes drugs			
MDI (lispro or aspart + glargine or detemir)	8		
CSII (lispro)	2		
Other drugs	0		

Table 1: Demographic and descriptive characteristics of non-obese Japanese patients with T1DM

The results are expressed as mean ± SE. All samples were collected in the overnight fasting. SUIT=250 x fasting serum immunoreactive C-peptide / (fasting plasma glucose- 3.43). Differences between the means of variables in two groups were evaluated statistically by chi square or unpaired t tests with or without Welch's correction. Two tailed values of P<0.05 were defined as statistically significant.

T1DM: Type 1 Diabetes Mellitus; BMI: Body Mass Index; SUIT: Secretory Units of Islet in Transplantation; MDI: Multiple Daily Insulin Injection Therapy; CSII: Continuous Subcutaneous Insulin Infusion.

Second, based on GLP-1 secretion, non-obese Japanese patients with T2DM (n=23) and control with NGT (n=13) (Table 2) were studied [18], in which non-obesity was based on criteria of the Japan Society for the Study of Obesity [7]. Diabetic patients were diagnosed by the WHO criteria [6] with $\geq 6.4\%$ of HbA1c (NGSP) (Table 2). Patients with insulin treatment were excluded from this study, because that insulin therapy may influence the p-GLP-1 levels [21]. Demographic characteristics of the non-obese participants are presented in Table 2. Patients and control subjects were matched by sex, age, and BMI. All patients had a mean of 10 years duration from discovering the disease (Table 2). Therefore, some patients had RP, RNP, NP as microvascular disturbance, CHD or CVD as macrovascular disturbance (Table 2). As they had ≥ 1.0 ng/mL of fasting s-CPR concentrations, they were mainly treated with oral drugs for hyperglycemia. Oral drugs were consisted of α -glycosidase inhibitors (α -GI), biguanide (BG), sulfonylurea (SU), thiazolidine (TZD) or combinations with them (Table 2). Also, some patients were treated with anti-hyperlipidemic, anti-hypertensive or anti-hyperuricemic drugs (Table 2). Control had <6.1% of HbA1c (NGSP). None of the participants had a history of gastrointestinal diseases, anemia, or impaired liver function and none were receiving any other medications.

Variables	Type 2	Control	P value
Number	23	12	
Age (years)	58 \pm 2.7	52 \pm 2.0	0.1850
Gender (males/females)	18/5	4/8	0.0240
BMI (kg/m ²)	21 \pm 0.3	21 \pm 0.5	1.0000
HbA1c (%) (NGDP)	9.1 \pm 0.30	5.6 \pm 0.01	<0.0010
Fasting plasma glucose (mg/dL)	151 \pm 9	97 \pm 2	<0.0010
Serum immunoreactive insulin (μ U/mL)	5.2 \pm 0.6	4.3 \pm 0.6	0.1690
HOMA-R	1.9 \pm 0.2	1.0 \pm 0.2	0.0010
HOMA- β (%)	25.1 \pm 3.4	80.1 \pm 5.1	0.0010
Serum immunoreactive C-peptide (ng/mL)	1.7 \pm 0.1	1.4 \pm 0.1	0.0730
Plasma immunoreactive active GLP-1 (pmol/L)	4.0 \pm 0.5	2.3 \pm 0.9	0.1020
Ratio of plasma immunoreactive active GLP-1/fasting plasma glucose ($\times 10^6$)	0.4 \pm 0.01	0.5 \pm 0.10	0.0250
Duration of diabetes from discovery (year)	9.2 \pm 1.5		
Chronic complications			
Microangiopathy	8		
Retinopathy (NDR/SDR//PPDR/PDR)	19/1/0/3		
Peripheral neuropathy	8		
Nephropathy (Normo/Micro/Macro)	17/5/1		
Macroangiopathy (asymptomatic)	3		

Therapy			
Diabetes drugs (α -GI/BG/SU/TZD)	1/15/12/12		
Lipid-lowering drugs	2		
Hypotensive drugs	5		
Anti-coagulant drugs	3		

Table 2: Demographic and descriptive characteristics of non-obesity patients with T2DM

Data are means \pm SE. Each value was collected in the morning after overnight fasting. HOMA-R and HOMA- β were calculated by the equations of fasting serum immunoreactive insulin (s-IRI) \times fasting plasma glucose (FPG)/405 and fasting s-IRI \times 360/ (FPG - 63), respectively. Differences between the means of variables in two groups were statistically evaluated by chi square or unpaired t tests. Two-tailed values of P<0.05 were defined as statistically significant. Control had normal glucose tolerance.

T2DM: Type 2 Diabetes Mellitus; BMI: Body Mass Index; NGSP: National Glycohemoglobin Standard Program; HOMA-R: Homeostasis Model Assessment- Insulin Resistance; HOMA- β : Homeostasis Model Assessment- Beta cell function; NDR: Non-Diabetic Retinopathy; SDR: Simple Diabetic Retinopathy; PPDR: Pre-Proliferative Retinopathy; PDR: Proliferative Retinopathy; Normo: Normoalbuminuria; Mico: Microalbuminuria; Macro: Macroalbuminuria; α -GI: Alpha-Glucosidase Inhibitors; BG: Biguanide; SU: Sulfonylurea; TZD: Thiazolidinedione

Third, based on GLP-1 secretion, obese Japanese patients with T2DM (n=24) and control with NGT (n=12) were included in this study [19]. The presence of obesity was based on criteria of the Japan Society for the Study of Obesity [7]. Diabetic patients were diagnosed according to the WHO criteria [8]. Patients receiving insulin treatment were excluded, because insulin therapy may influence p-GLP-1 levels [21]. Demographic characteristics of subjects participated were shown in Table 3. Patients and control subjects were matched by sex, age, and BMI. As the patients had a mean of 9 years passed the diagnosis of diabetes, some had RP, RNP or NP as microvascular disturbance, and CHD or CHD as macrovascular disturbance. As they had ≥ 1.0 ng/mL of fasting s-CPR concentrations, they were mainly treated with oral drugs for hyperglycemia. Oral drugs were consisted of α -GI, BG, SU, TZD, or combinations of these agents. Some patients were also receiving anti-hyperlipidemic, anti-hypertensive or anti-hyperuricemic drugs. Control was recruited from obese persons with NGT with <6.5% of HbA1c (NGSP) [9]. None of the participants had a history of gastrointestinal diseases, anemia, or impaired liver function and none were receiving any other medications.

Variables	Type 2	Control	P value
Number	24	12	
Age (y)	57 \pm 1	54 \pm 2	0.4790
Gender (males/females)	16/8	8/4	0.2670
BMI (kg/m ²)	28.6 \pm 0.7	27.5 \pm 0.5	0.2050
HbA1c (%) (NGSP)	8.0 \pm 0.3	5.7 \pm 0.1	<0.0010

Fasting plasma glucose (mg/dL)	158 ± 7	101 ± 2	<0.0010
Serum immunoreactive insulin (μU/mL)	7.8 ± 1.0	8.1 ± 1.5	0.0670
HOMA-R	2.9 ± 0.4	2.1 ± 0.1	0.6620
HOMA-β (%)	33.1 ± 5.7	80.1 ± 15.1	0.0110
Serum immunoreactive C-peptide (ng/mL)	2.3 ± 0.2	2.1 ± 0.3	0.5750
Plasma immunoreactive active GLP-1 (pmol/L)	2.9 ± 0.3	3.8 ± 0.9	0.2400
Ratio of plasma immunoreactive active GLP-1/fasting plasma glucose (x10 ⁶)	0.34 ± 0.001	0.71 ± 0.13	0.0010
Time passed diagnosis of diabetes (year)	9.0 ± 1.3		
Chronic complications			
Microangiopathy	6		
Retinopathy (NDR/SDR//PPDR/PDR)	18/3/2/1		
Peripheral neuropathy	2		
Nephropathy (Normo/Micro/Macro)	20/2/2		
Macroangiopathy (asymptomatic)	3		
Therapy			
Diabetes drugs (α-GI/BG/SU/TZD)	6/18/4/22		
Lipid-lowering drugs	10		
Hypotensive drugs	11		
Anti-hyperuricemic drugs	3		
Anti-coagulant drugs	3		

Table 3: Demographic and descriptive characteristics of obesity patients with T2DM

Data are means ± SE. Each value was collected in the morning after overnight fasting. HOMA-R and HOMA-β were calculated by the equations of fasting serum immunoreactive insulin (s-IRI) × fasting plasma glucose (FPG)/405 and fasting s-IRI × 360/(FPG - 63), respectively. Differences between the means of variables in two groups were statistically evaluated by chi square or unpaired t tests. Two-tailed values of P<0.05 were defined as statistically significant. Control had normal glucose tolerance.

T2DM: Type 2 Diabetes Mellitus; BMI: Body Mass Index; NGSP: National Glycohemoglobin Standard Program; HOMA-R: Homeostasis Model Assessment- Insulin Resistance; HOMA-β: Homeostasis Model Assessment- Beta cell function; NDR: Non-Diabetic Retinopathy; SDR: Simple Diabetic Retinopathy; PPDR: Pre-Proliferative Retinopathy; PDR: Proliferative Retinopathy; Normo: Normoalbuminuria; Mico: Microalbuminuria; Macro: Macroalbuminuria; α-GI: Alpha-Glucosidase Inhibitors; BG: Biguanide; SU: Sulfonylurea; TZD: Thiazolidinedione

Fourth, based on GIP secretion, Japanese patients with T1DM (n=15), and non-obese and obese Japanese patients with T2DM (n=29) were studied [20]. This study did not have a control. The presence of non-obesity and obesity was based on criteria of the Japan Society for the Study of Obesity [7]. Diabetic patients were diagnosed according to the WHO criteria [8]. Demographic characteristics of subjects participated were shown in Table 4. As the patients had more than mean of 9 years passed the diagnosis of diabetes, some had RP, RNP or PN as microvascular disturbance, and CHD or CVD as macrovascular disturbance. Patients with T1DM were treated with MDI or CSII (Table 4) and they with T2DM were mainly treated with oral drugs for hyperglycemia. Oral drugs were consisted of α-GI, BG, SU, TZD, or combinations of these agents (Table 4). Some patients were also receiving anti-hyperlipidemic, anti-hypertensive or anti-hyperuricemic drugs (Table 4).

Variables	Group 1	Group 2	P value
Type of diabetes mellitus	T1DM	T2DM	
Number	15	29	0.0010
Age (years)	54 ± 4	59 ± 2	0.0001
Gender (male/female)	8/7	17/12	0.9880
BMI (kg/m ²)	23.0 ± 0.7	27.0 ± 0.9	0.0010
HbA1c (%) (NGSP)	7.5 ± 0.6	8.7 ± 0.3	0.0001
Fasting plasma glucose (mg/dL)	148 ± 16	164 ± 9	0.0001
Serum immunoreactive C-peptide (ng/mL)	0.3 ± 0.01	1.8 ± 0.2	0.0001
SUIT	0.4 ± 0.1	5.4 ± 2.3	0.0001
Plasma immunoreactive total GIP (pmol/L)	7.3 ± 0.9	12.7 ± 1.4	0.0001
Ratio of plasma immunoreactive total GIP/fasting plasma glucose (x10 ⁶)	1.1 ± 1.2	1.4 ± 0.7	0.3391
Duration of diabetes from discovery (years)	19.0 ± 2.0	12.9 ± 1.8	0.0010
Chronic complications			
Microangiopathy	1	11	0.0100
Retinopathy (NDR/SDR//PPDR/PDR)	15/0/0/0	18/7/1/3	0.0100
Peripheral neuropathy	0	9	0.0100
Nephropathy (Normo/Micro/Macro)	14/1/0	20/9/0	0.0100
Macroangiopathy (asymptomatic)	0	4	0.0100
Therapy			
Diabetes drugs α-GI/BG/SU/TZD/Insulin	0/0/0/15	6/19/5/11/12	0.0010
Lipid-lowering drugs	0	6	0.0100
Hypertensive drugs	2	11	0.0100

Table 4: Demographic and descriptive characteristics varied in BMI of Japanese patients with T1DM and T2DM

Data are expressed as means \pm SE. Each value was collected in the morning after a 10-hour overnight fasting. SUII=250 x fasting serum immunoreactive C-peptide/(fasting plasma glucose-3.43). Differences between the means of variables in two groups were statistically evaluated by chi square or unpaired t tests. Two-tailed values of $P < 0.05$ were defined as statistically significant. Control had normal glucose tolerance.

T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus; BMI: Body Mass Index; NGSP: National Glycohemoglobin Standard Program; SUII: Secretory Units of Islet in Transplantation; NDR: Non-Diabetic Retinopathy; SDR: Simple Diabetic Retinopathy; PPDR: Pre-Proliferative Retinopathy; PDR: Proliferative Retinopathy; Normo: Normoalbuminuria; Mico: Microalbuminuria; Macro: Macroalbuminuria; α -GI: Alpha-Glucosidase Inhibitors; BG: Biguanide; SU: Sulfonylurea; TZD: thiazolidinedione.

Written informed consent was obtained from all subjects after informing them of the purpose and nature of the study. These studies were performed in accordance with the Declaration of Helsinki and with the approval of our hospital ethics committees.

Study design

The studies were randomized and cross-sectional, and conducted for 20 months from the beginning between from 10/1/2010 to 10/9/2011. After 10-hour overnight fast, subjects were placed in a seated position at 9:00 am with one cannula inserted into the cubital vein for blood sampling based on the method of Vilsbøll et al. [14]. TM (560 kcal) comprised of 60% carbohydrate, 23% fat and 17% protein based on JDS (10-11). Patients stopped taking all oral medications during the study, but continued the therapy with insulin.

Insulin-resistance and beta-cell function were assessed using homeostasis model assessment for HOMA-R and HOMA- β (%) calculated by the equations of [fasting serum immunoreactive insulin (s-IRI) level \times fasting PG (FPG) level]/405 and [fasting s-IRI level \times 360/(FPG level-63)], respectively [22]. When s-IRI value was not accurate due to production of antibody for IRI, SUII was calculated by the formula: 250 x fasting s-CPR/ (FPG-3.43) as previously described [23]. This has been as an index of beta-cell function using s-CPR in the islet transplanted patients with T1DM. Insulinogenic index was also calculated by the ratio of change in postprandial s-IRI /PG from baseline to 30 minutes after ingestion of TM based on a result obtained by the 75g-OGTT [10, 11]. Further, ratio of p-GLP-1/PG was calculated by the ratio of change in levels from baseline to 60 minutes after ingestion of TM.

As patients had a long duration of DM passed the diagnosis of diabetes, some had checked chronic complications as mentioned above.

Blood samples were collected in ice-cooled tubes from the inserted cannula immediately before, and 30 and 60 minutes after ingestion of TM. They were separated by centrifugation at 4°C for later determination of PG, s-IRI, s-CPR, p-GLP-1 and p-GIP levels. They were also used to measure HbA1c levels. Especially, blood samples for p-GLP-1 and p-GIP, and glucose were collected in ice-cooled vacutainers containing EDTA with 10 μ l DPP-IV inhibitor (diprotin) per mL of blood [14] and in vacutainers containing NaF, respectively.

Increment levels of PG, s-IRI, s-CPR, p-GLP-1 or p-GIP after ingestion of TM were calculated from the integrated areas under curves (AUC) from 0 to 60 minutes after ingestion of TM based on the

method of Vilsbøll et al. [14]. The AUC of GIP was presented as two responses. One is in early-phase (AUC of 0-30 minutes after ingestion of TM) and other is in late-phase (AUC of 0-60 minutes after ingestion of TM) [14].

Assay methods

HbA1c (JDS) was measured using Japanese JCCLS CRM-004 substance as a standard by high-performance liquid chromatography [25].

PG was measured by using oxidase method using commercial kit (Arkray Inc., Kyoto and Nittobo Medical Co., Tokyo, Japan). The sensitivity and the specificity were less than 3%.

S-IRI was measured by two-site sandwich immunoassay kit (Abbott, Co., Tokyo, Japan or Tosoh Co., Tokyo, Japan). S-CPR was measured by two-site sandwich immunoassay kit (Roche Diagnostics, Co., Tokyo, Japan or Tosho Co., Tokyo, Japan). Both lower detection limits were 1.0 μ U/mL for s-IRI and 0.2 ng/mL for s-CPR. The intra- and inter-assay coefficients of variation were both $< 5\%$. IRI assays showed 100%, 0.1% and 0.001% cross-reactivities with human insulin, human pro-insulin and human CPR, respectively. CPR assays showed 69% and 0% cross-reactivities with human pro-insulin and human insulin, respectively.

P-GLP-1 was measured at SRL, Inc. (Tokyo, Japan) with two-site sandwich immunoassay using a commercially available active GLP-1 Linco kit (Linco Research, MO, USA) [26] in unextracted samples as based on many literatures [26], although the JDS for Standardized Incretin Measurement was nowadays not approved [27]. The antibody provided with kit specifically recognizes the N-terminal region of active GLP-1 (7-36 and 7-37), but not other forms of GLP-1 (1-36,1-37, 9-36 and 9-37). The limit of detection for this assay was < 2.0 pmol/L. The intra- and inter-assay coefficients of variation were both $< 13\%$ [26].

P-GIP was measured at SRL, Inc. with two-site sandwich immunoassay using a total GIP Linco kit, as previously reported [28-29], in unextracted samples as based on the JDS for Standardized Incretin Measurement [27]. The antibody provided with kit specifically recognizes human GIP (1-42) and GIP (3-42), but not other forms of oxyntomodulin, GLP-1 and GLP-2.

Statistical methods

Results were expressed as means \pm SE. Differences between means of basal variables, insulinogenic index or AUC in groups were evaluated statistically by chi square or unpaired t tests with or without Welch's correction.

Repeated measures one-way or two-way analysis of variance (ANOVA) test was used to determine how response was affected by ingestion of TM in groups. Following one-way test or two-way test, a Bonferroni's test was used as a post hoc test to compare variables in groups.

The correlation coefficient (r) and the direction and magnitude of correlation in samples based on p-GIP were statistically analyzed using Pearson correlation analysis and the linear least squares method. Two-tailed P-values < 0.05 were considered as statistically significant.

Analysis was performed using GraphPad Prism version 5.04 (GraphPad Software, CA, USA).

Results

Baseline of number participated, age, BMI, gender, duration of disease, HbA1c, FPG, s-IRI, s-CPR, HOMA-R, HOMA-β, SUIT, chronic complications and therapies

In baseline, the means of number participated and age were significantly ($P < 0.0001$) lower in group with T1DM than in group with T2DM, but there was no significant difference in mean of BMI between groups with DM and controls (Tables 1-3). However, mean of BMI was significantly ($P < 0.0001$) lower in non-obese group with T2DM than in obese group with T2DM, Mean in ratio of male to female (gender) was significantly greater in non-obese group with T2DM than in control (Table 2). Mean of diabetes duration was significantly ($P < 0.001$) longer in group with T1DM than in group with T2DM. Meanwhile, means of HbA1c and FPG were significantly ($P < 0.0001$) higher in groups with DM than in controls (Tables 1-3). There was no significant ($P < 0.0001$) difference in mean of s-IRI or s-CPR level between groups with T2DM and controls (Tables 2 and 3), whereas mean of s-CPR level was significantly lower in group with T1DM than in groups with T2DM and control, although s-IRI level was not evaluated in group with T1DM owing to production of IRI antibody with immunoreactive method. Instead, mean of SUIT was significantly ($P < 0.0001$) lower in group with T1DM than in groups with T2DM and control. However, mean of HOMA-R was significantly ($P < 0.0001$) lower in non-obese group with T2DM than in obese group with T2DM, whereas it was significantly higher in non-obese or obese group with T2DM than in groups with T1DM and controls (Tables 2 and 3). Mean of HOAMA-β was significantly lower in all groups with T2DM than in controls (Tables 2 and 3). The mean of incidence in micro- or macroangiopathy was significantly ($P < 0.0001$) lower in group with T1DM than in group with T2DM, Number of patients treated with Insulin therapy was observed significantly ($P < 0.001$) greater in group with T1DM than in group with T2DM.

Postprandial in PG, s-IRI, and s-CPR

In postprandial levels following ingestion of TM, means of PG at each time in groups with T1DM and T2DM were significantly higher than in controls (Figures 1-6), whereas there was no significant difference in them between groups with non-obesity and obesity in T2DM.

The data are expressed as means ± SE. The mean body mass index (BMI) was not significantly different between groups. This test was performed in the following 10 hr of overnight fasting. The patients were treated with multiple daily injections or continuous subcutaneous insulin infusion using insulin analogues, who had received sc injection of bolus rapid-acting insulin analogues before ingestion of the test meal. The dotted lines indicate the limit level of detection for IRI, CPR, and active-GLP-1 assays. To compare the difference, a Bonferroni's test was used as a post hoc test after repeated measures one way ANOVA test or two-way ANOVA test. Two-tailed values of $P < 0.05$ were defined as statistically significant (**** $P < 0.0001$, *** $P < 0.001$ and ** $P < 0.01$ vs. before ingestion of the test meal, and #### $P < 0.0001$, ### $P < 0.001$ and ## $P < 0.01$ vs. control subjects).

There were no significant differences in means of s-IRI and s-CPR at each time between non-obese group with T2DM and control (Figure 2), although they were significantly ($P < 0.001$) lower in non-obese group with T2DM than in obese group with T2DM.

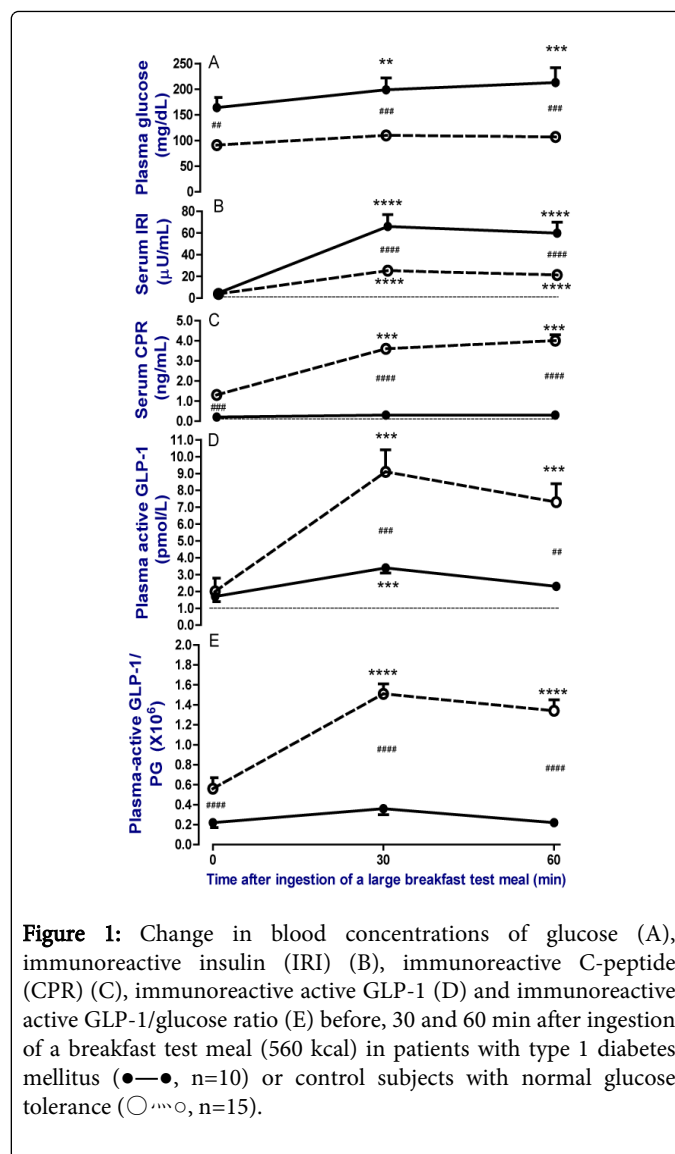
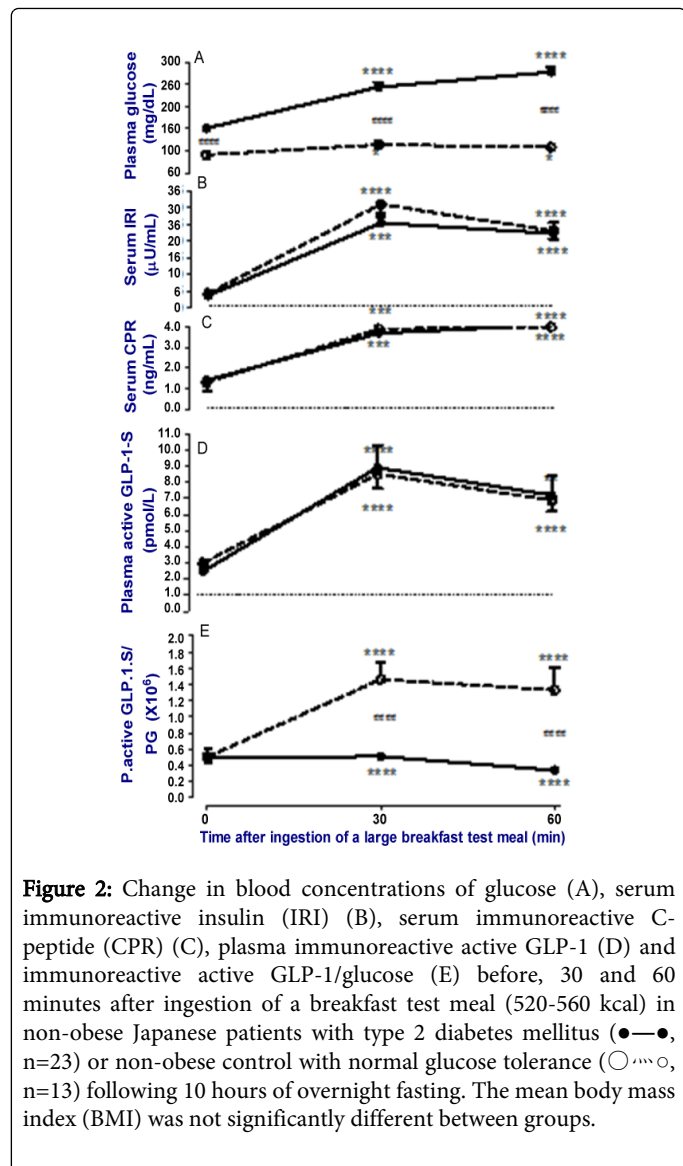


Figure 1: Change in blood concentrations of glucose (A), immunoreactive insulin (IRI) (B), immunoreactive C-peptide (CPR) (C), immunoreactive active GLP-1 (D) and immunoreactive active GLP-1/glucose ratio (E) before, 30 and 60 min after ingestion of a breakfast test meal (560 kcal) in patients with type 1 diabetes mellitus (●—●, n=10) or control subjects with normal glucose tolerance (○---○, n=15).

The data are expressed as means ± SE. The patients were treated with diet, exercise or/and oral medical drugs for hyperglycemia before ingestion of the test meal, but were stopped to receive the all medications during the study.

The dotted lines indicate the limit level of detection for IRI, CPR, and active-GLP-1 assays. To compare the difference, a Bonferroni's test was used as a post hoc test after repeated measures one-way ANOVA test or two-way ANOVA test. Two-tailed values of $P < 0.05$ were defined as statistically significant (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ vs. before ingestion of the test meal, and #### $P < 0.0001$, ### $P < 0.001$ and ## $P < 0.01$ vs. control subjects).

Mean of insulinogenic index was significantly ($P < 0.001$) lower in groups with T2DM than in control (Figures 1-3), and it was also significantly ($P < 0.03$) lower in non-obese group with T2DM than in obese group with T2DM. Mean of SUIT was significantly ($P < 0.001$) lower in group with T1DM than in control.

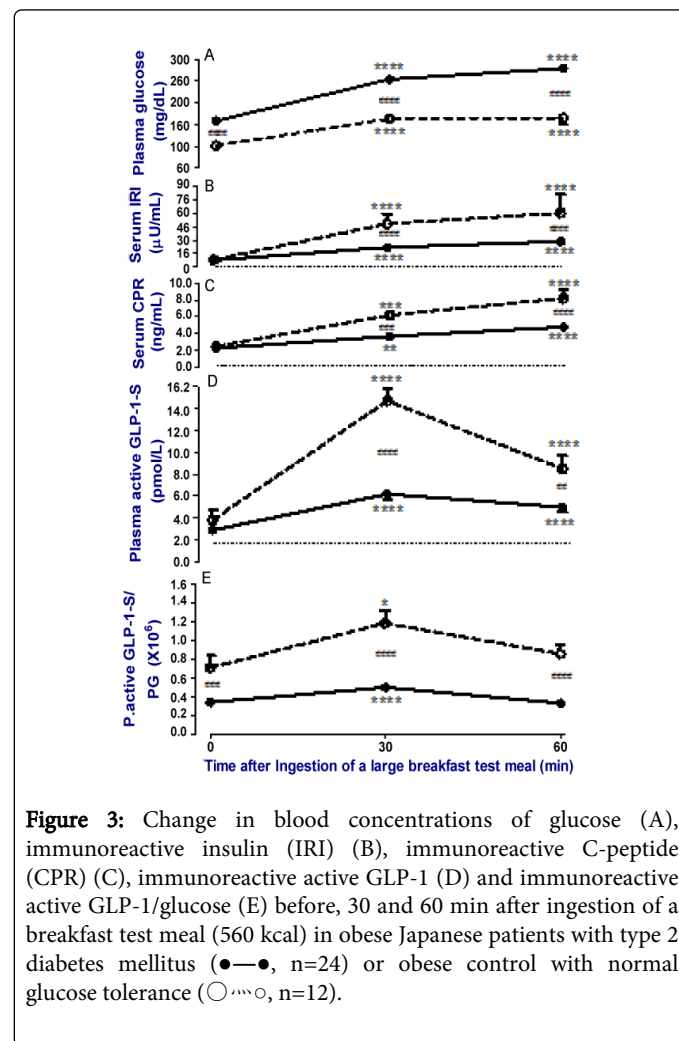


The data are expressed as means ± SE. The mean body mass index (BMI) was not significantly different between groups. This test was performed in the following 10 hr of overnight fasting. The taking all drugs in the subjects before and during the ingestion of test meal was stopped. The dotted lines indicate the limit level of detection for IRI, CPR, and active-GLP-1 assays. To compare the difference, a Bonferroni's test was used as a post hoc test after repeated measures one way ANOVA test or two-way ANOVA test. Two-tailed values of $P < 0.05$ were defined as statistically significant (**** $P < 0.0001$, *** $P < 0.001$ and * $P < 0.05$ vs. before ingestion of the test meal, and ### $P < 0.0001$, ## $P < 0.001$ and # $P < 0.01$ vs control subjects).

All patients did not complain the side effect of TM and there were no alterations in events of chronic complications and therapies for various diseases.

AUC in BMI, PG, s-CPR, HOMA-R, and HOMA-β

On the AUC, mean BMI was significantly ($P < 0.001$) higher in obese group with T2DM than in group with T1DM or non-obese group with T2DM, and mean of PG level was significantly higher in group with DM than in control. Mean of AUC in s-CPR was significantly ($P < 0.001$) lower in group with T1DM or non-obese group with T2DM than in obese group with T2DM or controls. Further, mean of AUC in HOMA-R was significantly ($P < 0.001$) lower in non-obese group with T2DM than in obese group with T2DM, and mean of AUC in HOMA-β was also significantly ($P < 0.0001$) lower in group with T1DM or non-obese T2DM than in group with obese T2DM or controls. There were no alterations in events of chronic complications and therapies for various diseases.



GLP-1

On based on GLP-1, at baseline levels there were no significant differences between groups with T1DM and T2DM, and controls (Tables 1-3). In postprandial levels following ingestion of TM, p-GLP-1 levels at each time were significantly ($P < 0.001$) lower in group with T1DM or obese group with T2DM than in controls (Figures 1 and 3), but they were significantly ($P < 0.0001$) higher in non-obese group with T2DM than in obese group with T2DM, although there was no significant difference between non-obese groups with T2DM and control (Figure 2). Mean of AUC in p-GLP-1 was significantly ($P < 0.001$) lower in group with T1DM or obese group with T2DM than

in non-obese group with T2DM and controls. However, mean ratio of p-GLP-1/FPG was significantly ($P<0.001$) lower in groups with DM in spite of types of DM than in control (Tables 1-3) and mean of AUC (0-60) in ratio of p-GLP-1/PG was also significantly lower in groups with DM than in control (Figures 1-3).

GIP

In based on GIP secretion, the mean of it at baseline was significantly ($P<0.001$) lower in group with T1DM than in group with T2DM. After postprandial levels following ingestion of TM, means of p-GIP levels were significantly sharply increased in groups with T1DM and T2DM (Figures 4 and 5). Mean AUC of p-GIP was significantly ($P<0.001$) higher in obese group with T2DM than in non-obese groups with T1DM and T2DM, and was insignificantly different between non-obese groups with T1DM and T2DM. However, there was no significant difference in ratio of p-GIP/FPG between groups with T1DM and T2DM (Table 4). Mean ratio of p-GIP/ PG after TM were also significantly increased sharply in group with T1DM or non-obese group with T2DM, but gradually increased in obese group with T2DM (Figures 4 and 5). Mean AUC (0-60) in ratio of p-GIP/PG were similar to those of p-GIP in non-obese or obese group with T2DM.

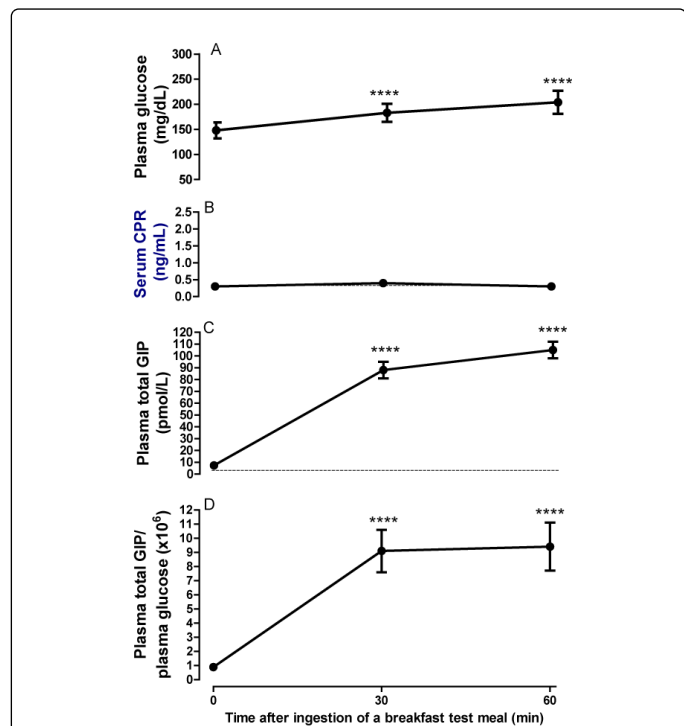


Figure 4: Change in blood concentrations of glucose (A), immunoreactive C-peptide (CPR) (B), immunoreactive total GIP (C) and immunoreactive total GIP/ glucose (D) before, and at 30 and 60 minutes after ingestion of a breakfast test meal (560 kcal) in Japanese patients with type 1 diabetes mellitus (●—●, n=15) following 10 hours of overnight fasting.

The data are expressed as means ± SE. The patients were treated with diet, exercise, and daily injections of insulin analogues. They had received a subcutaneous injection of insulin analogues before ingestion of the test meal. The dotted lines indicate the limit level of detection for CPR and total-GIP assays. To compare the difference, a

Bonferroni test was used as a post hoc test before and after repeated measures of one-way ANOVA test. Two-tailed values of $P<0.05$ were defined as statistically significant ($****P<0.0001$ vs. before ingestion of the test meal).

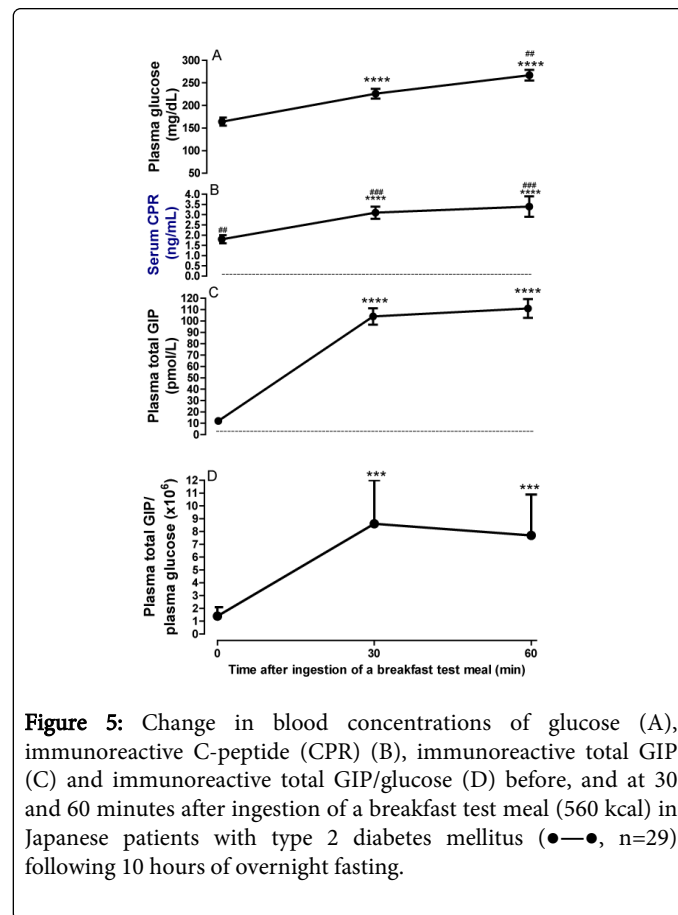


Figure 5: Change in blood concentrations of glucose (A), immunoreactive C-peptide (CPR) (B), immunoreactive total GIP (C) and immunoreactive total GIP/glucose (D) before, and at 30 and 60 minutes after ingestion of a breakfast test meal (560 kcal) in Japanese patients with type 2 diabetes mellitus (●—●, n=29) following 10 hours of overnight fasting.

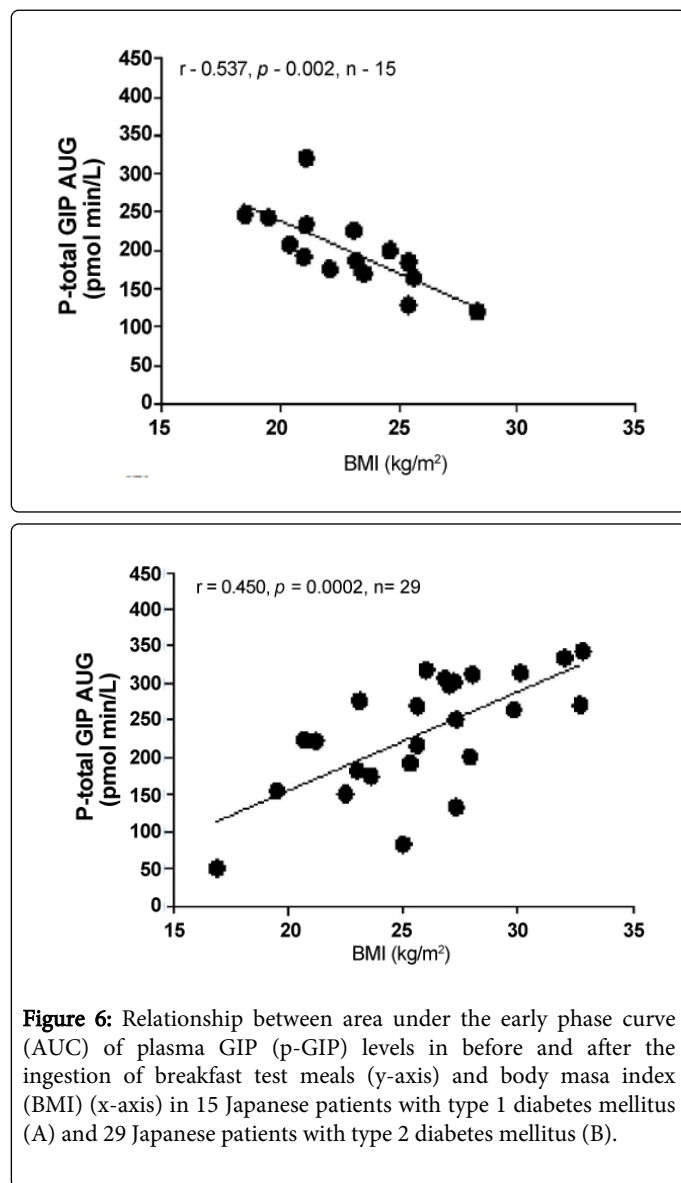
The data are expressed as means±SE. The patients were treated with diet, exercise, and/or oral pharmacotherapy, and/or daily injections of insulin analogues. They had not received oral drugs but received a subcutaneous injection of insulin analogues before ingestion of the test meal. The dotted lines indicate the limit level of detection for CPR and total-GIP assays. To compare the difference, a Bonferroni test was used as a post hoc test of one-way ANOVA test for before and after test meal and of two-way ANOVA test for before and after test meal. Two-tailed values of $p<0.05$ were defined as statistically significant ($****P<0.0001$ vs. before ingestion of the test meal, and $###P<0.001$, $##P<0.02$ and $#P<0.05$ vs. type 1 diabetes mellitus).

The AUC (0-30) of early phase was significant negatively and significant positively related to BMI in groups with T1DM and with T2DM, respectively (Figure 6).

Test meal consisted of 560 kcal (17% protein, 23% fats, and 60% carbohydrate).

Prior to testing, all patients underwent a 10-hour overnight fast. During the study, patients stopped all oral medications, but received subcutaneous injections of insulin analogues. The early phase of AUC was calculated using p-GIP levels from baseline to 30 minutes after the test meal. The patients (n=6) treated with α-glycosidase inhibitor were

excluded because that the drugs influence BMI and secretion of GIP. The correlation coefficient (r) and the direction and magnitude of correlation in samples were statistically analyzed using Pearson correlation analysis with the linear least squares method. Two-tailed values of $P < 0.05$ were considered to be statistically significant.



Discussion

We examined p-GLP-1 or p-GIP levels before and after ingestion of TM in patients with DM.

In based on GLP-1, when we compared diabetic patients and controls, fasting and postprandial p-GLP-1 levels were similar to both groups as the reports by Lee et al. [15] and Kozawa et al. [16]. Lee et al. showed that there were no significant differences in means of fasting and post prandial levels after TM in p-GLP-1 levels measured by using the same kit as this study between non-obese Japanese patients with T2DM and control [15]. On the other hand, Kozawa et al. reported that there were no low levels of p-GLP-1 in obese Japanese patients with T2DM [16], whereas Vilsbøll et al. and others reported that

postprandial levels after TM in p-GLP-1 were low in obese European patients with T2DM in comparison with control [14]. Although the reason for discrepancy in Japanese and European patients is unclarified, the differences may be due to several factors.

First, patients in this study had various degrees of BMI. Certainly, several studies have indicated a negative association between p-GLP 1 levels and BMI in patients with T2DM [30-32]. Non-obesity and obesity were defined by values of BMI, which was based on criteria of non-obesity as a BMI < 25 kg/m² and obesity as a BMI ≥ 25 kg/m² by the Japan [7], whereas in the U.S.A. and the Europe, ≥ 30.0 kg/m² in BMI are defined as obesity [7]. Therefore, the difference may be explained by the different values of BMI. Also, insulin-resistance increased with degree of BMI [19]. In this study, p-GLP-1 secretion was lower in obese patients with T2DM, who had a strong insulin-resistance, than in non-obese patients with T2DM, whereas they were not impaired in control with obesity, who had a strong insulin-resistance. Accordingly, GLP-1 may be related to DM and an impaired secretion of GLP-1 may be due to genetic factor [33-41] in patients with T2DM, irrespective of race.

Second, in patients with T1DM there was no insulin-resistance, supporting that they had normal HOMA-R, although insulin secretion was defected, while patients with T2DM had insulin-resistance and low insulin secretion, which was confirmed by high HOMA-R, low HOMA- β and low insulinogenic index. GLP-1 secretion after TM in this study was lower in Japanese patients with T1DM than in control, which is the opposite of others researchers [14-16], but it was significantly higher in obese patients with T2DM than in non-obese patients with T2DM or in patients with T1DM. This study shows that secretion of GLP-1 in patients with DM may be different between types of DM.

Third, the patients showed significant hyperglycemia as HbA1c and BG values in comparison with controls when fasting and test meal was ingested. Therefore, as the hyperglycemia may increase the DPP-4 activity in patients with DM [42], the difference of GLP-1at baseline and postprandial levels after following TM between Japanese and European patients with DM may be explained by the hyperglycemia. However, there was no significant difference in hyperglycemia between Japanese and European patients. Accordingly, the difference may not be explained by the hyperglycemia alone.

Fourth, duration of disease, medications for hyperglycemia, dyslipidemia, hypertension or hyperuricemia and diabetic complications may influence on the difference. The patients reported by Lee et al. had newly diagnosis as DM without using such drugs, and had no such complications [15]. Also, there was no statistical difference in AUC of incretin secretion and duration of DM between the study reported by Lee et al. and this study. Further, SU [43] or TZD [44] did not decrease secretion of GLP-1, although it is not known whether it influences secretion of GIP, while α -GI [45-47] or BG [48] may enhance secretion of GLP-1. Further, it was not known that anti-hyperlipidemic, anti-hypertensive or anti-hyperuricemic medicines influence secretion of GLP-1 and GIP [49]. Some diabetic complications, especially autonomic neuropathy may decrease incretin effect [50]. However, there was no statistical association between p-GLP-1 and p-GIP levels and autonomic neuropathy [30] as this study. Therefore, the factors may not influence incretin secretion and may be not related to the difference.

However, this study indicates that neither in non-obesity, obesity, types of DM, duration of diseases, hyperglycemia, chronic

complications, nor therapy for DM, ratio of p-GLP-1/PG was significantly lower in Japanese patients with T1DM and T2DM than in controls, indicating that DM may be related to an impaired GLP-1 secretion.

In based on GIP, mean in basal level was significantly lower in patients with T1DM than in those with T2DM. After postprandial levels following ingestion of TM, mean of p-GIP levels was significantly increased sharply in patients with T1DM and T2DM as reports previously [14-16]. Mean AUC of p-GIP was significantly higher in obese patients with T2DM than in non-obese patients with T2DM as well as with T1DM, and was insignificantly different between non-obese patients with T1DM and with T2DM. However, mean ratio of p-GIP/ FPG was no significantly different between patients with T1DM and T2DM in spite of obesity, but mean ratio of p-GIP/PG after TM was gradually increased in obese patients with T2DM alone.

The AUC in early phase was significant negatively and positively related to BMI in groups with T1DM and with T2DM, respectively. Although the limitation of this study is having no value of p-GIP in control, this study indicated that GIP secretion may have a function of insulin resistance [51] in patients with T2DM as well as a function of obesity [52-56]. Further, it is uncertain that the secretion is due to genetic factors as GLP-1 secretion or not [57,58].

In conclusion, our results indicated that basal and postprandial levels of p-GLP-1 after ingesting TM in Japanese patient with T1DM and T2DM in spite of non-obesity or obesity are similar to those of control, but basal and postprandial levels in ratio of p-GLP-1/PG in patients with T1DM or T2DM are low in comparison with controls. AUC of p-GIP in early-phase was negatively related to BMI in patients with T1DM, and positively related to BMI in patients with T2DM. Japanese patients with T2DM in spite of obesity may have a low secretion of GLP-1, which may be due to genetic factors. As obese persons with NGT, who had no impaired secretion of GLP-1, showed that there was no DM, a presence of person with MHO is true through the research of incretin secretion. Risk factors for DM are important to diagnose T2DM in spite of obesity and race.

Acknowledgments

We are grateful to the staffs of clinical nursing, clinical laboratory technician and clinical dietitian of Nagaoka Red Cross Hospital and Niigata Medical Center for their valuable technical assistances.

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This article was originally published in a special issue, entitled: "**Obesity & Diabetes**", Edited by Prof. Ouliana Ziouzenkova, Ohio State University, USA