Clinical study

Blood lactate and *MATE* genotype data were collected from patients, as described previously by Toyama *et al.* (2010). Of the 48 patients, lactate data were obtained from 29 patients receiving 250 mg of metformin in the morning. All were inpatients at the Department of Diabetes and Clinical Nutrition, Kyoto University Hospital. Patients were given metformin hydrochloride tablets (Melbin®, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) continuously for the treatment of diabetes mellitus. Blood lactate levels were measured at 0, 4 and 9 h after the administration of metformin using Lactate Pro (Arkray). This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by Kyoto University Graduate School and the Faculty of Medicine, Ethics Committee.

Uptake experiments

The detail of the methods used for the metformin uptake experiments were as described previously (Tanihara *et al.*, 2007). Briefly, HEK293 cells (American Type Culture Collection CRL-1573, Manassas, VA, USA) were transfected with mouse Mate1, mouse Oct1, and mouse Oct2 cDNAs using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37°C for 1 or 2 min with incubation medium (pH = 7.4) containing [^{14}C]-metformin in the presence or absence of 5 mM 1-methyl-4-phenylpyridinium (MPP). Unlabelled metformin was added to [^{14}C]-metformin to obtain the final concentrations. The concentration-dependence of metformin transport by mouse Mate1, mouse Oct1 and mouse Oct2 was analysed by use of the Michaelis–Menten equation: $V = V_{max}[S] / (K_m + [S]) + K_d \cdot [S]$, where V is the transport rate, V_{max} is the maximal transport rate, [S] is the concentration of metformin, K_m is the Michaelis–Menten constant and K_d is a diffusion constant.

Analytical methods

Metformin concentrations in plasma, liver, kidney and skeletal muscle were determined by HPLC, as described previously (Kimura *et al.*, 2005). The methods used for sample pretreatment were as described in detail previously (Tsuda *et al.*, 2009). AST and ALT were measured by Transaminase C II-Test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer's directions. Creatinine and BUN were measured by the LabAssay creatinine and UN *B*-test Wako (Wako Pure Chemical Industries Ltd.), respectively.

Materials

Metformin hydrochloride was obtained from Wako Pure Chemical Industries Ltd. [^{14}C]-metformin hydrochloride (54 mCi·mmol^{-1}) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). MPP iodide was purchased from Sigma Aldrich (St. Louis, MO, USA). All other compounds used were of the highest purity available.

Statistical analysis

Data are expressed as the mean ± SEM. Data were analysed statistically using Student's unpaired *t*-test or one-way ANOVA with Dunnett's multiple comparison test. The data were analysed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Continuous oral administration of metformin in mice

Metformin-induced lactic acidosis was evaluated using *Mate1*$^{+/+}$, *Mate1*$^{+/-}$ and *Mate1*$^{-/-}$ mice. It was confirmed that the volume of drinking water without metformin was not different amongst the three genotypes. In the presence of metformin, the volume of drinking water was decreased in *Mate1*$^{-/-}$ mice, resulting in a reduction in the mean daily dose of metformin in *Mate1*$^{-/-}$ mice (7.0 mg·day^{-1}) compared with *Mate1*$^{+/+}$ (12.0 mg·day^{-1}) and *Mate1*$^{+/-}$ mice (11.4 mg·day^{-1}) (Figure 1A). Plasma concentrations of metformin were within the range for clinical use (*Mate1*$^{+/+}$, 1.4 ± 0.2; *Mate1*$^{+/-}$, 2.1 ± 0.3; *Mate1*$^{-/-}$, 4.2 ± 0.3 μg·mL^{-1}). There was no significant difference in body weight change amongst the three genotypes (Figure 1B). At 7, 14 and 21 days after metformin treatment, a higher blood lactate level was observed in *Mate1*$^{-/-}$ mice compared with those in *Mate1*$^{+/+}$ mice (Figure 1C). pH and HCO_3^- levels were significantly lower in *Mate1*$^{-/-}$ mice (Figure 1D and E). At day 21, there were no significant differences in AST, ALT and BUN between *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice (Table 1). In *Mate1*$^{+/-}$ mice, all parameters were comparable to those in *Mate1*$^{+/+}$ mice (Figure 1C–E).

Blood lactate levels in diabetic patients

Because of the limited number of patients examined (n = 48), no homozygous *MATE* variant carrier was found in this clinical study. Heterozygous *MATE* variants have little influence on the metformin plasma concentration and oral clearance as described previously (Toyama *et al.*, 2010). In the present study, the effect of metformin on blood lactate levels was examined in 29 patients receiving 250 mg of metformin. Four heterozygous *MATE*-variant carriers were found; one patient carried *MATE1*-L125F variant, two carried *MATE1*-G64D variant and one carried *MATE2-K*-G211V variant. As expected, there was no significant difference in blood lactate levels between the two groups (Figure 2).

Single oral administration of metformin in mice

Based on the present data, no functional difference in the *MATE1* heterozygous genotype in comparison with the wild-type was observed *in vivo*, and therefore, we focused on the difference between the *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice. In the long-term administration experiment, the pharmacokinetics of metformin in *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice could not be rigorously evaluated because of the different dosages between the two groups. Therefore, the blood lactate levels and the pharmacokinetics of metformin were investigated in *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice after a single oral dose. In the vehicle-treated mice, there was no significant difference between these parameters in *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice. In metformin-treated mice, two *Mate1*$^{-/-}$ mice died and blood lactate levels were significantly high in *Mate1*$^{-/-}$ mice compared with

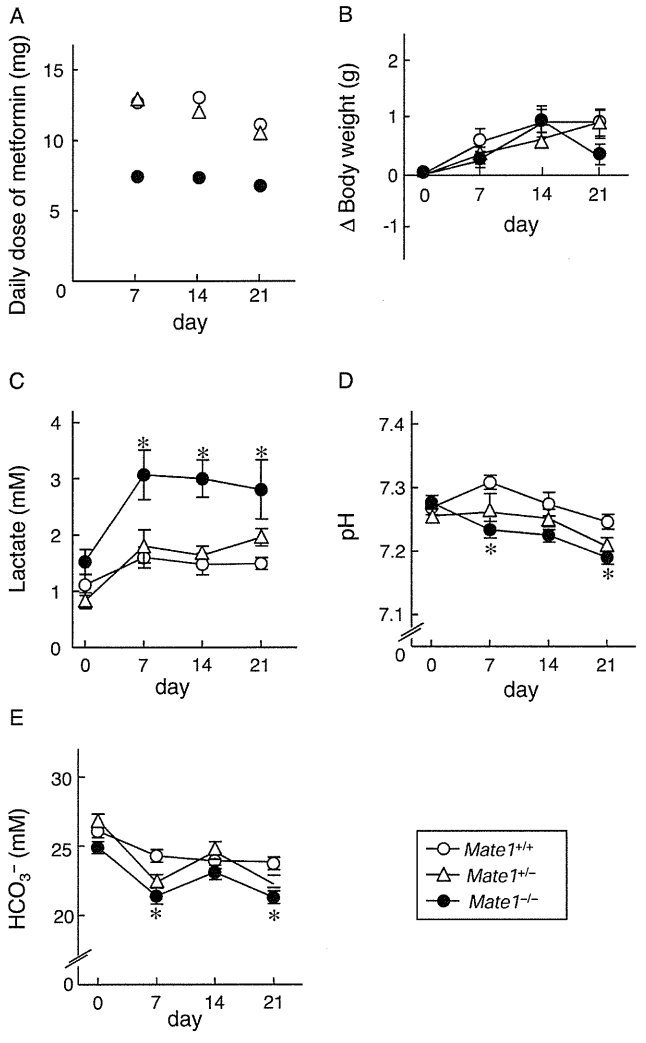

Figure 1

Long-term toxicity of metformin in *Mate1*$^{+/+}$, *Mate1*$^{+/-}$ and *Mate1*$^{-/-}$ mice. *Mate1*$^{+/+}$ (n = 9), *Mate1*$^{+/-}$ (n = 9) and *Mate1*$^{-/-}$ (n = 11) mice were given 3 mg·mL^{-1} metformin in drinking water for 21 days. The mean daily dose of each 7 days (A), body weight change from baseline (B), blood lactate level (C), pH (D) and HCO_3^- levels (E) were determined at 0, 7, 14 and 21 days after metformin treatment. Blood samples were collected under anaesthesia in 4 h-fasted mice. Blood lactate, pH and HCO_3^- levels were measured by i-STAT. Each point represents the mean ± SEM. *P < 0.05, significantly different from *Mate1*$^{+/+}$ mice at each day.

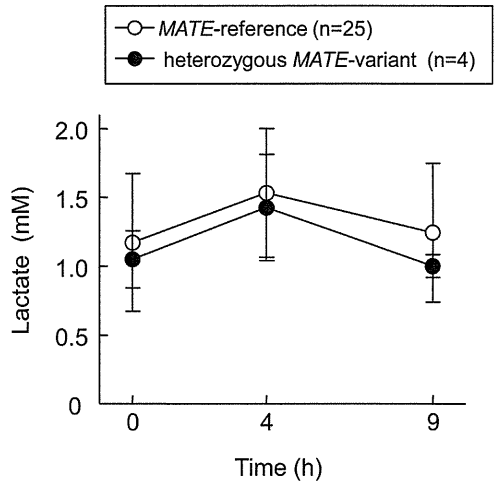

Figure 2

Lactate concentration–time profile in diabetic patients. Metformin was administered to patients in the *MATE*-reference group (n = 25) and heterozygous *MATE*-variant group (n = 4). Blood lactate levels were measured by Lactate Pro at 0, 4 and 9 h after the oral administration of metformin. One *MATE1*-L125F variant carrier, two *MATE1*-G64D variant carriers and one *MATE2-K*-G211V variant carriers were found in this study. Each point represents the mean ± SD.

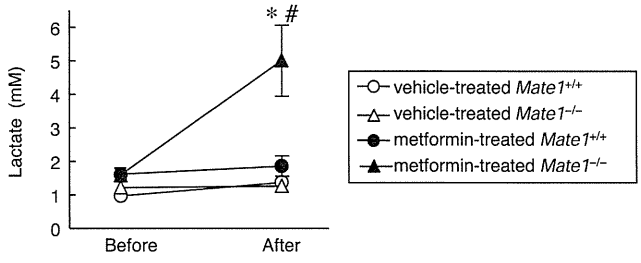

Figure 3

Blood lactate level in vehicle- or metformin-treated *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice after a single dose of metformin. All mice were given 400 mg·kg^{-1} metformin via oral gavage. Blood lactate levels were determined before and 24 h after the oral administration of metformin in vehicle-treated *Mate1*$^{+/+}$ mice (n = 6), vehicle-treated *Mate1*$^{-/-}$ mice (n = 7), metformin-treated *Mate1*$^{+/+}$ mice (n = 7) and metformin-treated *Mate1*$^{-/-}$ mice (n = 10). Each point represents the mean ± SEM. *P < 0.05, significantly different from vehicle-treated mice with the same genotype. $^{\#}P$ < 0.05, significantly different from metformin-treated *Mate1*$^{+/+}$ mice.

Mate1$^{+/+}$ mice (Figure 3). Several other biochemical parameters were also evaluated (Table 2). The pH and HCO_3^- levels were lower in metformin-treated *Mate1*$^{-/-}$ mice than in metformin-treated *Mate1*$^{+/+}$ mice. In addition, the base excess of extracellular fluid as an indicator of metabolic acidosis was also significantly decreased in metformin-treated *Mate1*$^{-/-}$ mice compared with metformin-treated *Mate1*$^{+/+}$ mice.

Plasma concentrations of metformin were 15-fold higher in *Mate1*$^{-/-}$ mice than in *Mate1*$^{+/+}$ mice (Figure 4A). Metformin concentrations in the kidney, liver and skeletal muscle were also 21-, 69- and 44-fold higher in *Mate1*$^{-/-}$ than those in *Mate1*$^{+/+}$ mice, respectively (Figure 4B, D and F). In *Mate1*$^{-/-}$ mice, the apparent tissue-to-plasma concentration ratio (K_p) was higher in the liver and skeletal muscle, but not in the kidney (Figure 4C, E and G).

Metformin and lactate concentration–time profile in mice

To clarify the association between the PK/PD profile, we examined the metformin and lactate concentration–time profile in *Mate1*$^{-/-}$ mice. The blood lactate level increased later than the plasma concentration of metformin; the plasma

Table 1

Biochemical parameters in *Mate1*$^{+/+}$, *Mate1*$^{+/-}$ and *Mate1*$^{-/-}$ mice 21 days after metformin treatment

	Mate1$^{+/+}$	*Mate1*$^{+/-}$	*Mate1*$^{-/-}$
Hepatic function			
AST (IU·L^{-1})	24 ± 3	21 ± 2	21 ± 2
ALT (IU·L^{-1})	33 ± 4	23 ± 1	20 ± 1
Renal function			
Creatinine (mg·mL^{-1})	0.0035 ± 0.0001	0.0038 ± 0.0001	0.0044 ± 0.0001*
BUN (mg·mL^{-1})	0.22 ± 0.02	0.23 ± 0.02	0.22 ± 0.01
Acid-base balance			
Lactate (mM)	1.5 ± 0.1	2.0 ± 0.2	2.8 ± 0.6*
pH	7.24 ± 0.01	7.21 ± 0.02	7.19 ± 0.01*
HCO_3^- (mM)	24 ± 1	22 ± 1	21 ± 0*

Mate1$^{+/+}$ (n = 9), *Mate1*$^{+/-}$ (n = 9) and *Mate1*$^{-/-}$ mice (n = 11) were used.
*P < 0.05, significantly different from *Mate1*$^{+/+}$ mice.

Table 2

Biochemical parameters and body weight in vehicle- or metformin-treated *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice.

	Vehicle		Metformin	
	Mate1$^{+/+}$	*Mate1*$^{-/-}$	*Mate1*$^{+/+}$	*Mate1*$^{-/-}$
BW (g)	30 ± 1	27 ± 1	29 ± 1	27 ± 0
pH	7.32 ± 0.02	7.28 ± 0.03	7.29 ± 0.02	7.03 ± 0.10**$^{\#\#}$
pCO_2 (mmHg)	50 ± 3	51 ± 4	48 ± 3	39 ± 3*
pO_2 (mmHg)	109 ± 17	114 ± 14	121 ± 4.1	127 ± 12
BE_{ecf} (mM)	−1 ± 1	−3 ± 1	−4 ± 1	−16 ± 2***$^{\#\#\#}$
HCO_3^- (mM)	25 ± 1	24 ± 1	23 ± 1	12 ± 2***$^{\#\#\#}$
TCO_2 (mM)	27 ± 1	25 ± 1	24 ± 1	15 ± 2***$^{\#\#\#}$
sO_2 (%)	96 ± 2	97 ± 1	98 ± 0	95 ± 1
Na (mM)	148 ± 1	151 ± 1	148 ± 1	153 ± 1$^{\#\#}$
K (mM)	5.5 ± 0.5	4.7 ± 0.2	4.9 ± 0.2	5.2 ± 0.2
Cl (mM)	116 ± 1	120 ± 1	117 ± 1	131 ± 0***$^{\#\#\#}$
iCa (mM)	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.1
Glu (mg·mL^{-1})	2.03 ± 0.16	1.78 ± 0.13	1.76 ± 0.09	1.44 ± 0.10*$^{\#}$
Hct (%PCV)	43 ± 2	42 ± 1	39 ± 1	42 ± 1
Hb (g·mL^{-1})	0.15 ± 0.01	0.14 ± 0.00	0.13 ± 0.00	0.14 ± 0.00
AnGap (mM)	15 ± 1	15 ± 0	15 ± 1	16 ± 1
BUN (mg·mL^{-1})	0.24 ± 0.04	0.24 ± 0.01	0.19 ± 0.05	0.45 ± 0.11
Creatinine (mg·mL^{-1})	0.0034 ± 0.0010	0.0036 ± 0.0002	0.0030 ± 0.0001	0.0054 ± 0.0008

Vehicle-treated mice (*Mate1*$^{+/+}$, n = 6; *Mate1*$^{-/-}$, n = 7), metformin-treated mice (*Mate1*$^{+/+}$, n = 7; *Mate1*$^{-/-}$, n = 10).
*P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle-treated mice with the same genotype; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, significantly different from *Mate1*$^{+/+}$ mice.
BW, body weight; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; BE_{ecf}, base excess of extracellular fluid; TCO_2, total carbon dioxide; sO_2, saturation oxygen; iCa, ionized calcium; Glu, glucose; Hct, haematocrit; Hb, haemoglobin; AnGap, anion gap.

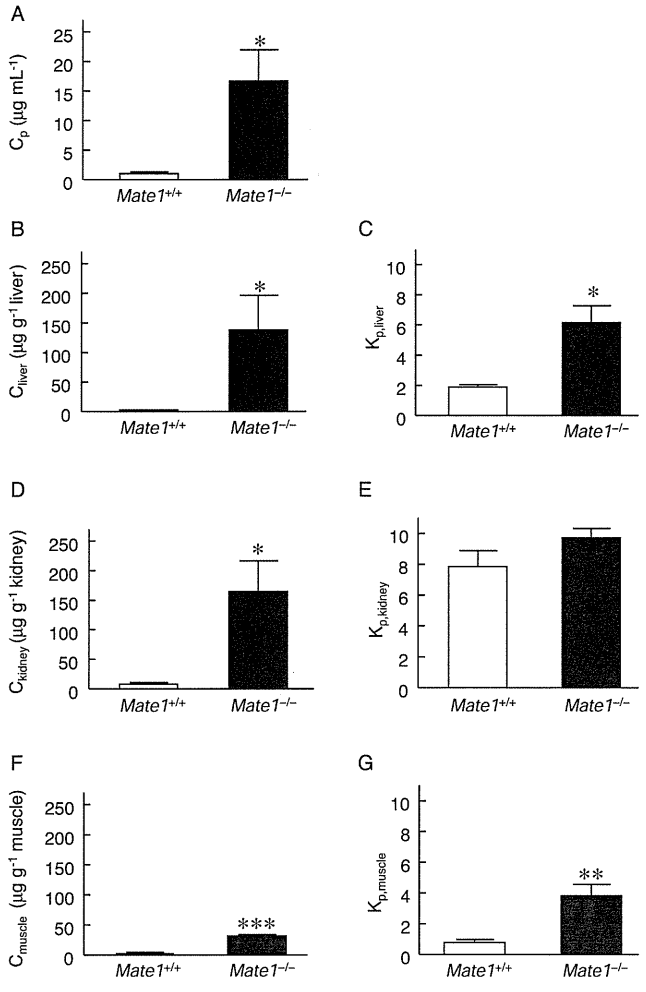

Figure 4

Pharmacokinetics of metformin in *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice after a single dose of metformin. In the same mice as shown in Figure 3, metformin concentrations in plasma (A), liver (B), kidney (D) and skeletal muscle (F) were determined by HPLC. K_p values in the liver (C), kidney (E) and skeletal muscle (G) were calculated by dividing the tissue concentration by the plasma concentration of metformin. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from *Mate1*$^{+/+}$ mice.

concentration of metformin and blood lactate level peaked at 2 and 4 h, respectively, after the oral administration metformin in these mice (Figure 5).

Concentration-dependence of [^{14}C]-metformin uptake by mouse Mate1, mouse Oct1 and mouse Oct2

We examined the characteristics of metformin transport by mouse Mate1, mouse Oct1 and mouse Oct2. The uptake of [^{14}C]-metformin by mouse Mate1, mouse Oct1 and mouse Oct2 was found to be concentration-dependent (Figure 6). The apparent K_m values of metformin transport by mouse Mate1-, mouse Oct1- and mouse Oct2-expressing cells were 0.3 ± 0.0, 1.8 ± 0.1 and 2.9 ± 0.4 mM, respectively. The V_{max}

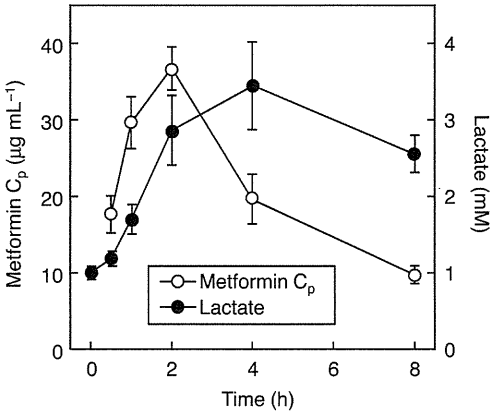

Figure 5

Lactate and metformin concentration–time profiles in *Mate1*$^{-/-}$ mice. Overnight-fasted mice were given 150 mg·kg^{-1} metformin (n = 8). Plasma concentration of metformin and blood lactate levels ere measured by HPLC and Lactate Pro respectively. Data represent mean ± SEM.

values were 5.3 ± 0.7 nmol·mg^{-1} protein·min^{-1}, 11.5 ± 2.3 nmol·mg^{-1} protein·2 min^{-1} and 18.5 ± 2.0 nmol·mg^{-1} protein·2 min^{-1} respectively.

Discussion

Genetic variants of drug transporters affect the pharmacokinetics of substrates and are involved in serious clinical outcomes. The organic anion transporting polypeptide 1B1 *(OATP1B1/SLCO1B1)* variant has been shown to increase plasma concentrations of simvastatin acid and is associated with statin-induced myopathy (Pasanen *et al.*, 2006; Link *et al.*, 2008). Our recent report suggested that the breast cancer resistant protein *(BCRP/ABCG2)* 421C>A variant causes the plasma concentration of sunitinib to increase and is associated with severe adverse effects to this drug such as skin reactions in the hands and feet (Mizuno *et al.*, 2010). In the case of metformin, a recent report demonstrated that MATE is involved in the excretion of metformin in humans by using the MATE inhibitor pyrimethamine (Kusuhara *et al.*, 2011). In addition, it was previously reported that the administration of metformin in the presence of pyrimethamine resulted in plasma concentrations of metformin 50 times higher than the therapeutic concentrations and also led to an increased blood lactate level (Ito *et al.*, 2010). In the present study, we focused on the clinical risk of metformin-induced lactic acidosis and carried out two experiments to examine the influence of *MATE* dysfunction on metformin-induced lactic acidosis in mice. On the continuous administration of metformin in drinking water, the mean plasma concentration of metformin in *Mate1*$^{+/+}$ mice was 1.4 μg·mL^{-1}, which is consistent with the clinical plasma concentration (Scheen, 1996; Toyama *et al.*, 2010). In *Mate1*$^{-/-}$ mice, the blood lactate level was significantly increased despite a lower dosage of metformin than in the *Mate1*$^{+/+}$ mice (Figure 1). After the same dose of metformin,

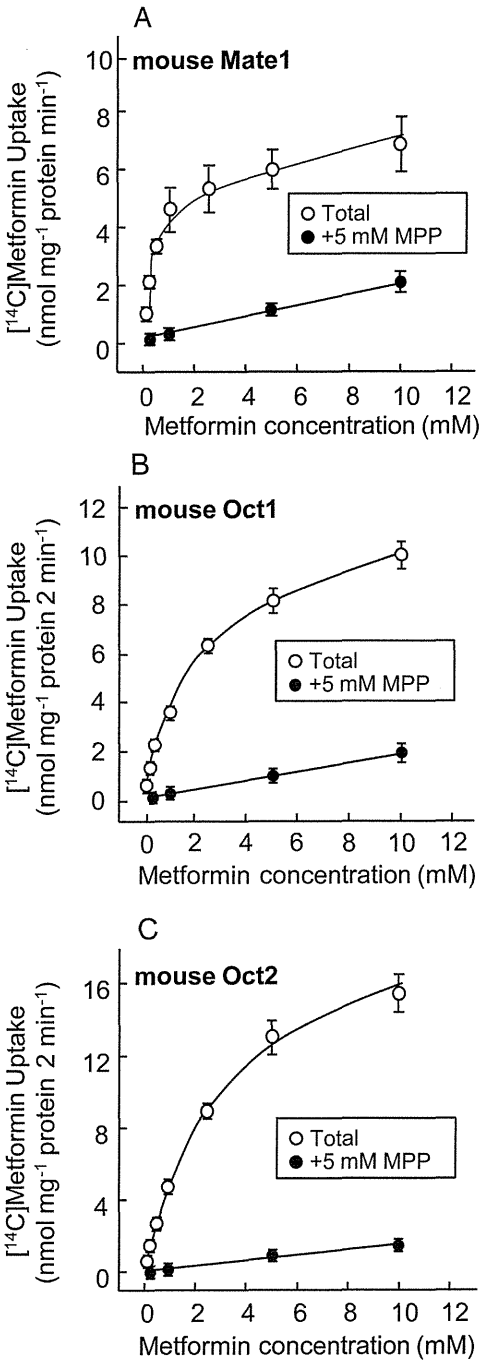

Figure 6

Concentration dependence of [^{14}C]-metformin uptake by mouse Mate1-, mouse Oct1- or mouse Oct2-expressing cells. The cells were incubated with [^{14}C]-metformin in the presence or absence of 5 mM MPP at 37°C for 1 min in mouse Mate1-expressing cells (A) and for 2 min in mouse Oct1- (B) or mouse Oct2-expressing cells (C). Data represent mean ± SEM.

administered as a bolus p.o., in *Mate1*$^{+/+}$ and *Mate1*$^{-/-}$ mice, severe lactic acidosis was observed in *Mate1*$^{-/-}$ mice, as shown by the levels of lactate, pH, HCO_3^- and several electrolytes, but not in the *Mate1*$^{+/+}$ mice (Figure 3 and Table 2). In addition, two of the *Mate1*$^{-/-}$ mice died after this acute metformin treatment, and the plasma creatinine concentration was also elevated in these metformin-treated *Mate1*$^{-/-}$ mice (Tables 1 and 2). These phenomena were reported in the patients with metformin-induced lactic acidosis (Misbin *et al.*, 1998; Tymms and Leatherdale, 1988; al-Jebawi *et al.*, 1998; Stades *et al.*, 2004). It was shown that the peak of blood lactate level followed the elevation of metformin concentration, and the high blood lactate level was maintained even though the metformin concentration was reduced immediately (Figure 5). These results suggest that metformin-induced lactic acidosis is caused by a completely dysfunctional MATE even when plasma concentration of metformin was within therapeutic range in *MATE*-reference carriers. Taken together, the data indicate that a dysfunctional *MATE* mutation could be a high risk factor for metformin-induced lactic acidosis.

In accord with our previous report on the pharmacokinetics of metformin, we found that in humans, the heterozygous *MATE* variant did not affect blood lactate levels (Figure 2) (Toyama *et al.*, 2010). In addition, the same results were obtained in *Mate1*$^{+/-}$ mice (Figure 1). This suggests that metformin-induced lactic acidosis is not just caused by the heterozygous *MATE* variant. On the other hand, there is no information about the blood lactate level in human homozygous *MATE* variant carriers due to low allelic frequencies of *MATE* variants with a loss of function (Chen *et al.*, 2009; Kajiwara *et al.*, 2009; Meyer zu Schwabedissen *et al.*, 2010). Metformin-induced lactic acidosis is also quite rare with 3 cases per 100 000 patient-years, but severe with a mortality up to 50% (Bailey and Turner, 1996; Misbin *et al.*, 1998). If possible, the nucleotide sequences of MATE should be determined in patients suffering from lactic acidosis after receiving metformin treatment.

The liver is a major pharmacological target organ for the action of metformin. In a previous report it was shown that the blood lactate level was elevated in *Oct1*$^{+/+}$ mice, but not in *Oct1*$^{-/-}$ mice, after metformin treatment (Wang *et al.*, 2003). Also hepatic concentrations of metformin were significantly lower in *Oct1*$^{-/-}$ mice than in *Oct1*$^{+/+}$ mice at similar plasma concentrations. Therefore, it was suggested that liver is the key organ responsible for metformin-induced lactic acidosis. In the present study we demonstrated that the hepatic concentration of metformin was 69-fold higher in *Mate1*$^{-/-}$ mice than in *Mate1*$^{+/+}$ mice, in spite of a 15-fold increase in the plasma concentration (Figure 4A and B). In *Mate1*$^{-/-}$ mice, the renal clearance of metformin was 18% of that in *Mate1*$^{+/+}$ mice, and the plasma concentration of metformin was higher (Tsuda *et al.*, 2009). In addition, it was reported that the MATE1 inhibitor pyrimethamine causes a 53 % reduction in hepatic clearance of metformin with respect to the liver concentration of metformin (Ito *et al.*, 2010). Taken together, these data indicate that a MATE1 dysfunction synergistically increased metformin accumulation in the liver due to the loss of urinary and biliary efflux of metformin, resulting in the development of lactic acidosis.

In addition to its effects in the liver, metformin, by activation of AMP-activated protein kinase (AMPK), also has a pharmacological effect in skeletal muscle (Zhou *et al.*, 2001). The AMPK activator AICAR has been reported to increase significantly the rate of lactate release within skeletal muscle

cells (Young *et al.*, 1996). A recent report showed that metformin was transported into skeletal muscle cells by OCT3 (Chen *et al.*, 2010). MATE1 is also expressed in skeletal muscle (Otsuka *et al.*, 2005) and in the present study it was found that the K_p value for metformin was increased in muscle from *Mate1*$^{-/-}$ mice (Figure 4G). Therefore, it is likely that MATE1 is responsible for metformin efflux from the skeletal muscle as well as in the kidney and liver.

Species differences exist in the MATE family (Yonezawa and Inui, 2011). The tissue distribution of the MATE family is reported to differ between humans and mice. MATE1 and MATE2-K are expressed in human kidney, whereas MATE2-K is not expressed in mice (Masuda *et al.*, 2006; Tsuda *et al.*, 2009). Therefore, *Mate1* knockout mice could represent a model of both MATE1 and MATE2-K deficiency in humans, because of similar affinities in mice and humans (Figure 6) (Tanihara *et al.*, 2007). The renal clearance of metformin in *Mate1*$^{+/-}$ mice was comparable with that in *Mate1*$^{+/+}$ mice despite the reduction of MATE1 expression (Toyama *et al.*, 2010). Taken together with the similar transport characteristics of MATE1 and MATE2-K (Tanihara *et al.*, 2007), it is assumed that the renal excretion of metformin was not affected in either homozygous *MATE1* or homozygous *MATE2-K* variant carriers. In the pharmacological targets such as liver and skeletal muscle, MATE1 was found but not MATE2-K (Yonezawa and Inui, 2011). In *Mate1*$^{-/-}$ mice, the hepatic and skeletal muscular K_p values for metformin were higher than those in the wild-type mice (Figure 4C and G). Therefore, it is anticipated that only the homozygous *MATE1* variant causes the elevation in metformin concentrations in the liver and skeletal muscle, even though renal excretion is not changed. In fact, in a previous case report, metformin-induced lactic acidosis was found to occur within the therapeutic concentration of metformin (Tymms and Leatherdale, 1988; al-Jebawi *et al.*, 1998). MATE1 is expressed in several tissues that are associated with metformin action, but MATE2-K is expressed only in the kidney (Yonezawa and Inui, 2011). Therefore, only the homozygous *MATE1* variant would affect the pharmacodynamics of metformin without a reduction in renal clearance.

In the present study, a dysfunction in MATE1 caused lactic acidosis after metformin treatment, accompanied by a reduction in the renal clearance of metformin and increase in hepatic accumulation. These findings indicate that MATE1 plays an important role in the pharmacodynamics of metformin. In conclusion, the homozygous *MATE* variant is one of the risk factors for metformin-induced lactic acidosis. These observations may provide new insights into the causes of metformin-induced lactic acidosis.

Acknowledgements

The authors are grateful to all the medical staff of the Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, especially to Dr Chizumi Yamada, Dr Kazuyo Fujita, Dr Akio Obara, Dr Norio Harada, Dr Kazutaka Nagai and Dr Shiho Takahara for excellent help. This study was supported by a grant-in-aid for Scientific Research (KAKENHI) from the Ministry of Education, Science, Culture, and Sports of Japan, and in part by the Nakatomi Foundation.

Conflicts of interest

None.

References

Bailey CJ, Turner RC (1996). Metformin. N Engl J Med 334: 574–579.

Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH (2009). Genetic variation in the multidrug and toxin extrusion 1 transporter protein influences the glucose-lowering effect of metformin in patients with diabetes: a preliminary study. Diabetes 58: 745–749.

Chen L, Pawlikowski B, Schlessinger A, More SS, Stryke D, Johns SJ *et al.* (2010). Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. Pharmacogenet Genomics 20: 687–699.

Chen Y, Teranishi K, Li S, Yee SW, Hesselson S, Stryke D *et al.* (2009). Genetic variants in multidrug and toxic compound extrusion-1, hMATE1, alter transport function. Pharmacogenomics J 9: 127–136.

Ito S, Kusuhara H, Kuroiwa Y, Wu C, Moriyama Y, Inoue K *et al.* (2010). Potent and specific inhibition of mMate1-mediated efflux of type I organic cations in the liver and kidney by pyrimethamine. J Pharmacol Exp Ther 333: 341–350.

al-Jebawi AF, Lassman MN, Abourizk NN (1998). Lactic acidosis with therapeutic metformin blood level in a low-risk diabetic patient. Diabetes Care 21: 1364–1365.

Kajiwara M, Terada T, Ogasawara K, Iwano J, Katsura T, Fukatsu A *et al.* (2009). Identification of multidrug and toxin extrusion (MATE1 and MATE2-K) variants with complete loss of transport activity. J Hum Genet 54: 40–46.

Kimura N, Masuda S, Tanihara Y, Ueo H, Okuda M, Katsura T *et al.* (2005). Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. Drug Metab Pharmacokinet 20: 379–386.

Kusuhara H, Ito S, Kumagai Y, Jiang M, Shiroshita T, Moriyama Y *et al.* (2011). Effects of a MATE protein inhibitor, pyrimethamine, on the renal elimination of metformin at oral microdose and at therapeutic dose in healthy subjects. Clin Pharmacol Ther 89: 837–844.

Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F *et al.* (2008). SLCO1B1 variants and statin-induced myopathy – a genomewide study. N Engl J Med 359: 789–799.

Masuda S, Terada T, Yonezawa A, Tanihara Y, Kishimoto K, Katsura T *et al.* (2006). Identification and functional characterization of a new human kidney-specific H^+/organic cation antiporter, kidney-specific multidrug and toxin extrusion 2. J Am Soc Nephrol 17: 2127–2135.

Meyer zu Schwabedissen HE, Verstuyft C, Kroemer HK, Becquemont L, Kim RB (2010). Human multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter: functional

characterization, interaction with OCT2 (SLC22A2), and single nucleotide polymorphisms. Am J Physiol Renal Physiol 298: F997–F1005.

Misbin RI, Green L, Stadel BV, Gueriguian JL, Gubbi A, Fleming GA (1998). Lactic acidosis in patients with diabetes treated with metformin. N Engl J Med 338: 265–266.

Mizuno T, Terada T, Kamba T, Fukudo M, Katsura T, Nakamura E *et al.* (2010). ABCG2 421C>A polymorphism and high exposure of sunitinib in a patient with renal cell carcinoma. Ann Oncol 21: 1382–1383.

Otsuka M, Matsumoto T, Morimoto R, Arioka S, Omote H, Moriyama Y (2005). A human transporter protein that mediates the final excretion step for toxic organic cations. Proc Natl Acad Sci U S A 102: 17923–17928.

Pasanen MK, Neuvonen M, Neuvonen PJ, Niemi M (2006). SLCO1B1 polymorphism markedly affects the pharmacokinetics of simvastatin acid. Pharmacogenet Genomics 16: 873–879.

Pearlman BL, Fenves AZ, Emmett M (1996). Metformin-associated lactic acidosis. Am J Med 101: 109–110.

Safadi R, Dranitzki-Elhalel M, Popovtzer M, Ben-Yehuda A (1996). Metformin-induced lactic acidosis associated with acute renal failure. Am J Nephrol 16: 520–522.

Scheen AJ (1996). Clinical pharmacokinetics of metformin. Clin Pharmacokinet 30: 359–371.

Stades AM, Heikens JT, Erkelens DW, Holleman F, Hoekstra JB (2004). Metformin and lactic acidosis: cause or coincidence? A review of case reports. J Intern Med 255: 179–187.

Tanihara Y, Masuda S, Sato T, Katsura T, Ogawa O, Inui K (2007). Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H^+-organic cation antiporters. Biochem Pharmacol 74: 359–371.

Toyama K, Yonezawa A, Tsuda M, Masuda S, Yano I, Terada T *et al.* (2010). Heterozygous variants of multidrug and toxin extrusions (MATE1 and MATE2-K) have little influence on the disposition of metformin in diabetic patients. Pharmacogenet Genomics 20: 135–138.

Tsuda M, Terada T, Mizuno T, Katsura T, Shimakura J, Inui K (2009). Targeted disruption of the multidrug and toxin extrusion 1 (Mate1) gene in mice reduces renal secretion of metformin. Mol Pharmacol 75: 1280–1286.

Tymms DJ, Leatherdale BA (1988). Lactic acidosis due to metformin therapy in a low risk patient. Postgrad Med J 64: 230–231.

Wang DS, Kusuhara H, Kato Y, Jonker JW, Schinkel AH, Sugiyama Y (2003). Involvement of organic cation transporter 1 in the lactic acidosis caused by metformin. Mol Pharmacol 63: 844–848.

Yonezawa A, Inui K (2011). Importance of the multidrug and toxin extrusion MATE/SLC47A family to pharmacokinetics, pharmacodynamics/toxicodynamics and pharmacogenomics. Br J Pharmacol 164: 1817–1825.

Young ME, Radda GK, Leighton B (1996). Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR-an activator of AMP-activated protein kinase. FEBS Lett 382: 43–47.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J *et al.* (2001). Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108: 1167–1174.

Insulin secretory capacity and insulin sensitivity in impaired fasting glucose in Japanese

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ABSTRACT

Aims/Introduction: Impaired fasting glucose (IFG) increases the risk of developing diabetes mellitus (DM). This study was carried out to characterize Japanese patients who have fasting glucose levels (FPG) between 100 and 109 mg/dL ($IFG_{100-109}$).

Materials and Methods: A total of 1383 Japanese participants were examined by oral glucose tolerance test. We compared insulin secretory capacity (insulinogenic index) and insulin sensitivity (ISI composite) of $IFG_{100-109}$/normal glucose tolerance (NGT; 100 ≤ FPG < 110 mg/dL and 2-h postchallenge glucose level (2-hPG) < 140 mg/dL) with NGT (100 mg/dL < FPG and 2-hPG < 140 mg/dL) and $IFG_{110-125}$/NGT (110 ≤ FPG < 126 mg/dL and 2-hPG < 140 mg/dL). In addition, $IFG_{100-109}$ patients were analyzed in three subgroups according to glucose intolerance by 2-hPG.

Results: Of the three categories of $IFG_{100-109}$, $IFG_{100-109}$/DM had the lowest insulinogenic index despite an ISI composite showing only a small decline from $IFG_{100-109}$/NGT through $IFG_{100-109}$/IGT (100 ≤ FPG < 110 mg/dL and 140 ≤ 2-hPG < 200 mg/dL) to $IFG_{100-109}$/DM (100 ≤ FPG < 110 mg/dL and 200 mg/dL < 2-hPG). By multiple regression analysis, the insulinogenic index showed a significant relationship with 2-h PG levels. Both insulinogenic index and ISI composite were decreased significantly from NGT through $IFG_{100-109}$/NGT to $IFG_{110-125}$/NGT.

Conclusions: Although impaired early-phase insulin secretion plays the more important role in the elevation of postchallenge glucose in $IFG_{100-109}$ patients, both impaired early-phase insulin secretion and decreased insulin sensitivity are involved in the deterioration of FPG in Japanese. In addition, insulin secretory defect and decreased insulin sensitivity already have begun in patients with $IFG_{100-109}$. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2012.00201.x, 2012)

KEY WORDS: Impaired fasting glucose, Insulin secretion, Insulin sensitivity

INTRODUCTION

The prevalence of type 2 diabetes is increasing dramatically throughout the world; early detection of developing glucose intolerance is important to delay and prevent the disease. The American Diabetes Association (ADA) has lowered the cut-off value of impaired fasting glucose (IFG) from 110 to 100 mg/dL in 2003. Subjects with fasting plasma glucose (FPG) from 100 to 109 mg/dL together with normal postchallenge glucose levels have been classified as normal glucose tolerance (NGT) by the criteria of the World Health Organization (WHO) and the Japanese Diabetes Society (JDS), but are classified as IFG by the 2003 ADA criteria[1–3]. Insulin secretory capacity and insulin sensitivity are regulated differently with regard to fasting and postchallenge plasma glucose[4,5], and therefore require separate evaluation. In addition, there are ethnic differences in the pathology of developing glucose intolerance. In our previous studies, impaired insulin secretion is shown to play an especially important role in both diabetic and pre-diabetic Japanese subjects[4,5], in contrast to the increasing insulin resistance that is the more important factor in Caucasian, Mexican American and Pima Indian populations[6,7]. In the present cross-sectional study, we analyzed 1383 Japanese patients with fasting plasma glucose between 100 and 109 mg/dL ($IFG_{100-109}$) to characterize and compare insulin secretory capacity, and insulin sensitivity of Japanese patients with $IFG_{100-109}$ in the development of glucose intolerance.

METHODS

Subjects

A total of 1835 Japanese patients undergoing 75-g oral glucose tolerance test (OGTT) as a result of positive urine glucose test, ≥5.5% glycated hemoglobin (HbA_{1c}) level, ≥100 mg/dL fasting plasma glucose (FPG) level, and family history of diabetes at initial examination for medical check-up at Kyoto University

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Received 4 August 2011; revised 24 November 2011; accepted 1 December 2011

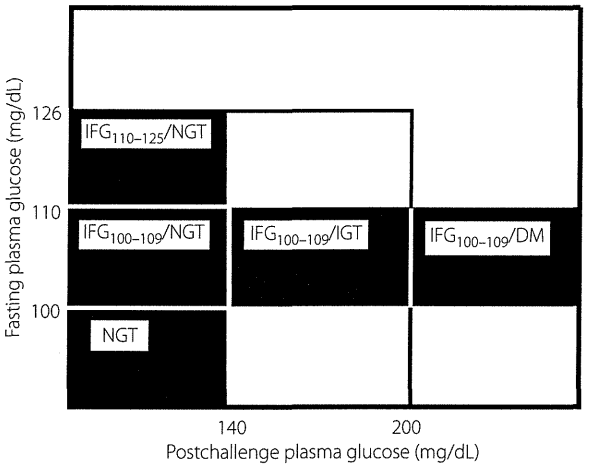

Figure 1 | Subgroups of impaired fasting glucose. DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

Hospital, Ikeda Hospital, Kansai Electric Power Hospital, Kansai Health Management Center, Center for Preventive Medicine of St. Luke's International Hospital and Kyoto Preventive Medical Center from 1993 to 2009 were examined. Originally, 358 patients who had hypertension, hepatic or renal dysfunction, endocrine or malignant disease, or history of heavy exercise, gastrectomy, or medication known to affect glucose metabolism were excluded from 2193 patients. Among the 1835 patients, 470 patients were excluded because of fasting glucose levels ≥ 126 mg/dL and postchallenge glucose levels ≥ 140 mg/dL with FPG ≥ 110 mg/dL and FPG < 100 for the present study, and 1383 patients were included. The study was designed in compliance with the ethics regulations of the Helsinki Declaration, and the study protocol was approved by the ethics committee of St. Luke's International Hospital.

Standard OGTT with 75-g glucose was given according to the National Diabetes Data Group recommendations[8], which require patients to fast overnight for 10–16 h. Fasting, 0.5, 1, and 2-h blood samples were obtained for measurement of plasma glucose and serum insulin after oral administration of 75-g glucose. Blood samples for measurement of HbA_{1c}, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglyceride levels were collected after overnight fast.

The subjects that underwent 75-g OGTT were divided into two groups: one having normal postchallenge glucose (2-hPG < 140 mg/dL) and another having $IFG_{100–109}$ (100 ≤ FPG < 110 mg/dL). The subjects of each group also were divided into three groups as shown in Figure 1.

Group one had normal postchallenge glucose: NGT (n = 594); normal fasting glucose according to ADA (FPG < 100 mg/dL) with normal postchallenge glucose, $IFG_{100–109}$/NGT (n = 369); $IFG_{100–109}$ (100 ≤ FPG < 110 mg/dL) with normal postchallenge glucose, $IFG_{110–125}$/NGT (n = 160); and IFG (110 ≤ FPG < 126 mg/dL) with normal postchallenge glucose. Group two had $IFG_{100–109}$: $IFG_{100–109}$/NGT (n = 369); $IFG_{100–109}$ with normal postchallenge glucose, $IFG_{100–109}$/IGT (n = 225); $IFG_{100–109}$ with impaired glucose tolerance (IGT; 140 ≤ 2-hPG < 200 mg/dL), and $IFG_{100–109}$/diabetes mellitus (DM; n = 35); $IFG_{100–109}$ with postchallenge hyperglycemia (200 mg/dL ≤ 2-hPG; Figure 1).

Measurements

Plasma glucose level was measured by the glucose oxidase method using a Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured by two-site radioimmunoassay (Insulin Riabead II; Dainabot, Tokyo, Japan), as reported previously[9]. HbA_{1c} was measured by HLC-723G7 (Tosoh, Tokyo, Japan). Coefficients of variation (CVs) were 0.56% for plasma glucose, <7% for insulin and <2% for HbA_{1c}. Serum total cholesterol and triglycerides levels were measured as reported previously[10].

Blood samples were collected at 0, 30, 60 and 120 min after OGTT, and plasma glucose and serum insulin levels were measured for all subjects. Blood samples for measurements of HbA_{1c}, total cholesterol, HDL-C and triglycerides were drawn after an overnight fast. The HbA_{1c} value was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value calculated by the formula: HbA_{1c} (NGSP) = HbA_{1c} (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)[2].

Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index[11–13], and Insulin sensitivity was determined by an OGTT based on the formula for the composite of insulin sensitivity index (ISI composite)[14]. Insulinogenic index < 0.4 is considered in Japanese to be decreased early-phase insulin secretion according to the 'Report of The Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus' by the Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus[2]. The calculations were as follows:

$$\text{Insulinogenic index (II)} = (\text{Ins}_{0.5} - \text{Ins}_0[\text{pmol/L}])/(\text{Glu}_{0.5} - \text{Glu}_0[\text{mmol/L}])^{11–13}$$

$$\text{ISI composite} = 10{,}000/([\text{Glu}_0 \times \text{Ins}_0] \times [\text{mean Glu}_{0–120} \times \text{mean Ins}_{0–120}])^{0.5},^{14}$$

We compared these two indices between IFG subgroups and NGT as shown in Figure 1 in this cross-sectional study.

Statistical Analysis

All of the statistical analyses were carried out using SPSS version 19.0 (SPSS Japan, Tokyo, Japan). Clinical characteristics of the study subjects are described using mean ± standard deviation, and general analysis of variance (ANOVA) was carried out for

between-group comparisons. The differences were established by *post-hoc* Bonferroni test for multiple comparisons. *P*-values <0.05 were considered statistically significant. Multiple stepwise regression analysis was done using AUC-G and 2-h PG as the dependent variables, and age, body mass index (BMI), ISI composite and insulinogenic index as the independent variables among the $IFG_{100-109}$ subgroups. In addition, simple regression analysis was applied between insulinogenic index and area under the curve of glucose (AUC-G), and also between insulinogenic index and 2-hPG, as the insulinogenic index showed the strongest relationship with these two factors.

RESULTS

Comparison of NGT, $IFG_{100-109}$/NGT and $IFG_{110-125}$/NGT

Clinical Characteristics

Table 1 shows the clinical and metabolic characteristics of the 1123 Japanese patients classified with NGT, $IFG_{100-109}$/NGT and $IFG_{110-125}$/NGT. The age and BMI (mean ± standard error) of the total of the three groups were 51.3 ± 0.4 years and 22.9 ± 0.2, respectively. The mean age of the NGT group was significantly lower than that of the other two groups ($P < 0.05$). The differences in BMI were significant among the three groups ($P < 0.05$, respectively). There was no significant difference in triglycerides, total cholesterol or HDL-C, although HbA_{1c} showed significant differences among the three groups ($P < 0.001$, respectively).

Insulin Secretion

The insulinogenic indices of the three groups are shown in Figure 2a; NGT 0.48, $IFG_{100-109}$/NGT 0.39, $IFG_{110-125}$/NGT 0.27. The insulinogenic index in the NGT group was significantly higher than that in the other groups ($P < 0.05$). The insulinogenic index in the $IFG_{100-109}$/NGT group was <0.4.

Table 1 | Clinical and metabolic characteristics of NGT, $IFG_{100-109}$/NGT and $IFG_{110-125}$/NGT

	NGT	$IFG_{100-109}$/NGT	$IFG_{110-125}$/NGT
n	594	369	160
Age (years)	48.6 ± 0.6	54.2 ± 0.6*	54.7 ± 0.7*
BMI (kg/m^2)	22.2 ± 0.1	23.1 ± 0.2	24.9 ± 1.2*,**
FPG (mg/dL)	90.3 ± 0.3	104.1 ± 0.2*	114.8 ± 0.3*,****
2-h PG (mg/dL)	104.5 ± 0.8	112.8 ± 0.9*	112.5 ± 1.5*
Fasting insulin (pmol/L)	33.3 ± 0.7	37.5 ± 1.0*	40.2 ± 1.6*
HbA_{1c} (%)	5.3 ± 0.0	5.7 ± 0.0*	5.9 ± 0.0*,****
Triglycerides (mmol/L)	1.26 ± 0.06	1.23 ± 0.05	1.28 ± 0.10
Total cholesterol (mmol/L)	5.28 ± 0.06	5.34 ± 0.05	5.38 ± 0.09
HDL cholesterol (mmol/L)	1.61 ± 0.03	1.63 ± 0.03	1.62 ± 0.05

Data are mean ± standard error. BMI, body mass index; FPG, fasting plasma glucose; HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; PG, plasma glucose. *$P < 0.05$ (vs normal glucose tolerance [NGT]); **$P < 0.05$ (vs impaired fasting glucose $[IFG]_{100-109}$/NGT); ***$P < 0.001$ (vs $IFG_{100-109}$/NGT).

Insulin Sensitivity

Figure 2b shows the ISI composites: NGT 10.3, $IFG_{100-109}$/NGT 7.9, $IFG_{110-125}$/NGT 7.2. ISI composite in the NGT was significantly higher than in the other groups ($P < 0.001$).

Comparison of $IFG_{100-109}$/NGT, $IFG_{100-109}$/IGT and $IFG_{100-109}$/DM

Clinical Characteristics

Table 2 shows the clinical and metabolic characteristics of the 629 Japanese patients classified with $IFG_{100-109}$/NGT, $IFG_{100-109}$/IGT and $IFG_{100-109}$/DM. The age and BMI (mean ± standard error) of all of the patients were 55.2 ± 0.4 years and 23.5 ± 0.1, respectively. However, the BMI of $IFG_{100-109}$/NGT subjects were significantly lower ($P < 0.05$). There was a significant difference in age only between $IFG_{100-109}$/NGT and $IFG_{100-109}$/DM. The differences in triglycerides and total cholesterol were significant only between $IFG_{100-109}$/NGT and $IFG_{100-109}$/IGT ($P < 0.05$). There was no significant difference in HDL-C, except for HbA_{1c}, which showed significant differences among the three groups ($P < 0.001$, respectively).

Insulin Secretion

The insulinogenic indices of the three groups are shown in Figure 2c. The insulinogenic index showed a significant difference between $IFG_{100-109}$/NGT and the others ($P < 0.05$), and the insulinogenic index of $IFG_{100-109}$/DM (0.19) was nearly 50% compared with $IFG_{100-109}$/NGT (0.39). The insulinogenic index (II) in the $IFG_{100-109}$/NGT (0.39) group was <0.4.

Insulin Sensitivity

Figure 2d shows the ISI composite of the three groups. There was a significant difference only between $IFG_{100-109}$/NGT and $IFG_{100-109}$/IGT ($P < 0.001$), which showed a 9% decline from $IFG_{100-109}$/NGT (7.9) to $IFG_{100-109}$/DM (7.2).

Simple and Multiple Regression Analysis

We examined the relationship between the dependent variables AUC-G and 2-hPG, and the independent variables of age, BMI, ISI composite and insulinogenic index by multiple regression analysis. Insulinogenic index showed the strongest relationship with AUC-G and 2-hPG (β of 2 h-PG: age 0.11, BMI 0.10, II −0.215, ISI composite −0.164; and β of AUC-G: age 0.082, BMI 0.048, II −0.545, ISI composite −0.350). Figure 3a shows scattered plots of simple regression analysis of AUC-G with insulinogenic index and ISI composite. Insulinogenic index ($r = 0.454$, $P < 0.001$, *F*-value 16.3) had a stronger relationship with AUC-G than with ISI composite ($r = 0.232$, $P < 0.001$, *F*-value 35.7). Figure 3b shows scattered plots of simple regression analysis of 2-hPG with insulinogenic index and ISI composite. Insulinogenic index ($r = 0.165$, $P < 0.001$, *F*-value 17.5) was

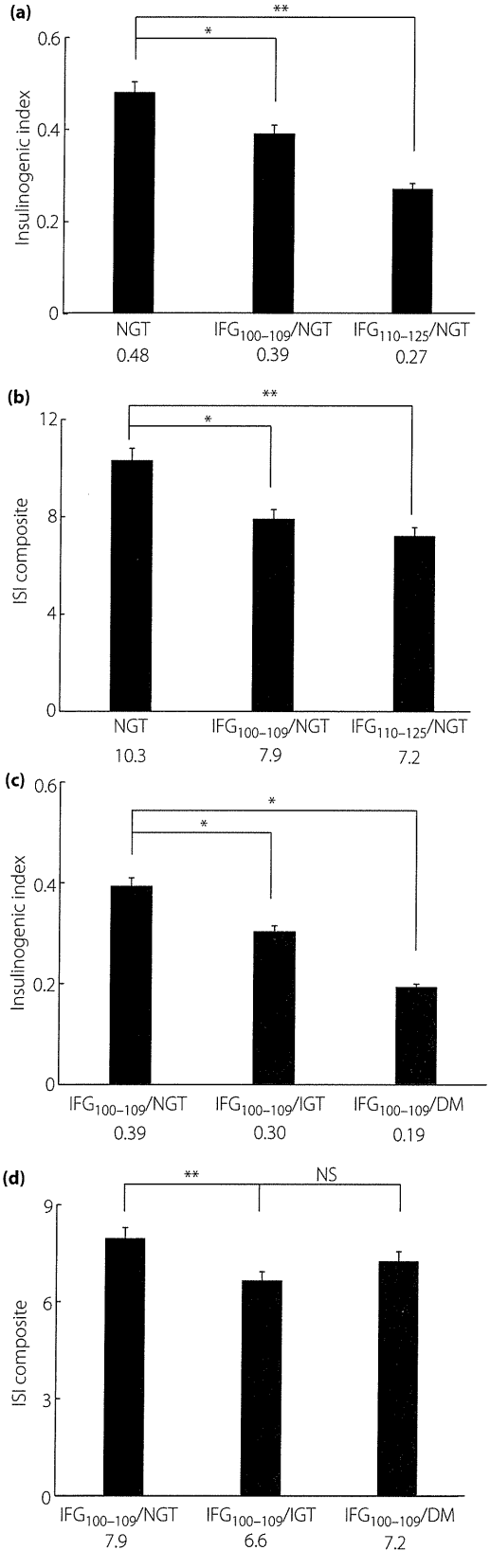

Figure 2 | Indices of insulin secretion and sensitivity. (a) Early-phase insulin secretion. Insulinogenic index in normal glucose tolerance (NGT) is highest and shows significant differences compared with the other two groups (NGT and impaired fasting glucose (IFG)$_{100–109}$/NGT: $P = 0.026$, NGT and IFG$_{110–125}$/NGT: $P \leq 0.001$). (b) Insulin sensitivity. Insulin sensitivity index (ISI) composite in NGT is significantly higher than the other groups. (NGT and IFG$_{100–109}$/NGT: $P \leq 0.001$, NGT and IFG$_{110–125}$/NGT: $P \leq 0.001$). (c) Early-phase insulin secretion. The difference of insulinogenic index is significant except between IFG$_{100–109}$/impaired glucose tolerance (IGT) and IFG$_{100–109}$/diabetes mellitus (DM; IFG$_{100–109}$/NGT and IFG$_{100–109}$/IGT: $P = 0.023$, IFG$_{100–109}$/NGT and IFG$_{100–109}$/DM: $P = 0.013$). (d) Insulin sensitivity. The difference of ISI composite is significant only between IFG$_{100–109}$/NGT and IFG$_{100–109}$/IGT (IFG$_{100–109}$/NGT and IFG$_{100–109}$/IGT: $P \leq 0.001$). *$P < 0.05$; **$P < 0.001$. NS, not significant.

Table 2 | Clinical and metabolic characteristics of IFG$_{100–109}$/NGT, IFG$_{100–109}$/IGT and IFG$_{100–109}$/DM

	IFG$_{100–109}$/NGT	IFG$_{100–109}$/IGT	IFG$_{100–109}$/DM
n	369	225	35
Age (years)	54.2 ± 0.6	56.3 ± 0.7	58.9 ± 1.9*
BMI (kg/m^2)	23.1 ± 0.2	24.0 ± 0.2	24.6 ± 0.8*
FPG (mg/dL)	104.1 ± 0.2	104.7 ± 0.3*	105.1 ± 0.5
2-h PG (mg/dL)	112.8 ± 0.9	161.8 ± 1.1**	223.8 ± 3.7**,***
Fasting insulin (pmol/L)	37.5 ± 1.0	44.6 ± 1.9**	41.2 ± 3.9
HbA$_{1c}$ (%)	5.7 ± 0.0	5.8 ± 0.0**	6.2 ± 0.1**,***
Triglycerides (mmol/L)	1.23 ± 0.05	1.64 ± 0.11**	1.35 ± 0.13
Total cholesterol (mmol/L)	5.34 ± 0.05	5.54 ± 0.07*	5.54 ± 0.17
HDL cholesterol (mmol/L)	1.63 ± 0.03	1.53 ± 0.04	1.59 ± 0.12

Data are mean ± standard error. BMI, body mass index; FPG, fasting plasma glucose; HbA$_{1c}$, glycated hemoglobin; HDL, high-density lipoprotein; PG, plasma glucose. *$P < 0.05$ (vs impaired fasting glucose [IFG]$_{100–109}$/normal glucose tolerance [NGT]); **$P < 0.001$ (vs IFG$_{100–109}$/NGT); ***$P < 0.001$ (vs IFG$_{100–109}$/impaired glucose tolerance [IGT]).

more related to 2-hPG than to ISI composite ($r = 0.150$, $P < 0.001$, F-value 14.5).

DISCUSSION

In the present study, we analyzed insulin secretory capacity and insulin sensitivity in a cross-sectional study in Japanese subjects who had IFG$_{100–109}$ (IFG$_{100–109}$; 100 ≤ FPG < 110 mg/dL). A reduction in the insulinogenic index, a measure of early-phase insulin secretion, has already begun in IFG$_{100–109}$/NGT subjects, for which the insulinogenic index was 0.39 (Figure 2a). The ISI composite, an index of systemic insulin sensitivity, is also decreased in the deterioration of FPG from NGT through IFG$_{100–109}$/NGT to IFG$_{110–125}$/NGT (Figure 2b). IFG$_{100–109}$/NGT subjects are classified into normal fasting glucose tolerance according to the 1998 WHO criteria and the criteria of the JDS,

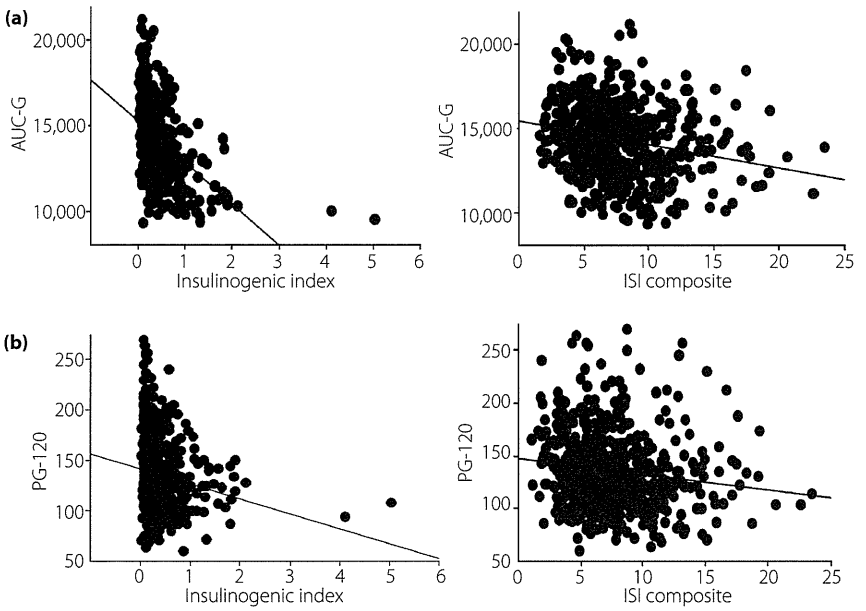

Figure 3 | (a) The relationship between area under the curve of glucose (AUC-G), and insulinogenic index and insulin sensitivity index (ISI) composite among the impaired fasting glucose ($IFG)_{100–109}$ groups. Insulinogenic index (r = 0.454, P < 0.001, F-value 16.3) was more related to AUC-G than ISI composite (r = 0.232, P < 0.001, F-value 35.7). (b) The relationship between 2-h postchallenge glucose level (PG-120), and insulinogenic index and ISI composite. Insulinogenic index (r = 0.165, P < 0.001, F-value 17.5) was more related to PG-120 than ISI composite (r = 0.150, P < 0.001, F-value 14.5).

although they are classified into IFG based on the 2003 ADA criteria. There are numerous studies of impaired fasting glucose that justify the new ADA criteria, but few studies focus on the borderline $IFG_{100–109}$ group regarding the relevance of insulin secretory capacity and insulin sensitivity in the development of disease. The present study showed deterioration of both early-phase insulin secretion and insulin sensitivity in $IFG_{100–109}$/NGT. To clarify the pathology, we evaluated $IFG_{100–109}$ subjects in three subgroups: $IFG_{100–109}$/NGT, $IFG_{100–109}$/IGT and $IFG_{100–109}$/DM. The mean insulinogenic indices were found to decrease from $IFG_{100–109}$/NGT through $IFG_{100–109}$/IGT to $IFG_{100–109}$/DM (0.39 [<0.40], 0.30 and 0.19, respectively), and those of $IFG_{100–109}$/IGT and $IFG_{100–109}$/DM were significantly lower than that of $IFG_{100–109}$/NGT. In contrast, the differences among ISI composites in $IFG_{100–109}$/NGT, $IFG_{100–109}$/IGT and $IFG_{100–109}$/DM were small. In addition, $IFG_{100–109}$/DM showed a stronger deterioration in insulin secretory capacity and insulin sensitivity than in $IFG_{110–125}$/NGT (P < 0.05). These results agree with those of our previous studies in Japanese subjects that show that impairment of early-phase insulin secretion plays the more important role in the deterioration of postchallenge glucose tolerance, whereas insulin resistance plays a lesser role[15].

It is notable that $IFG_{100–109}$/IGT and $IFG_{100–109}$/DM are included into $IFG_{100–109}$ if judged only by FPG. Although this study is not a population-based study, over 40% of the subjects with $IFG_{100–109}$ also were classified as IGT or DM. This suggests that DM, as well as IGT, might be overlooked when screening by FPG among subjects with $IFG_{100–109}$. IFG has been reported as high risk for developing type 2 diabetes. In a study of Japanese subjects, those with $IFG_{100–109}$ developed type 2 diabetes at twice the rate as NGT during 4 years[16]. Bonora *et al.*[17] found that diabetic incidence rates for 10 years of IGT, IFG and IFG/IGT were 3.9, 11 and 20.5 compared with that of NGT, respectively. Meigs *et al.*[18] found that 40% of isolated IFG progressed to diabetes. According to the Funagata study, the risk for type 2 diabetes in Japanese with isolated IFG is 20.5 compared with that in NGT[19]. IFG subjects, therefore, require checking of postchallenge plasma glucose levels by OGTT to prevent or delay diabetes.

Regarding insulin secretory capacity and insulin sensitivity, fasting and postchallenge plasma glucose levels are regulated differently[20]. Hyperglycemia in Japanese patients is typically as a result of factors that differ from those in other ethnic groups, impaired insulin secretion and sensitivity being most notable. We have previously reported that although impaired early-phase insulin secretion plays the more important role in deterioration from NGT through IGT to isolated postchallenge hyperglycemia in Japanese subjects[9], progression from NGT through IFG to isolated fasting hyperglycemia is associated with both impaired insulin secretion and decreased insulin sensitivity[21]. However, impaired insulin secretion is the more important factor in Japanese subjects, whereas increasing insulin resistance is the more important factor in Caucasians,

Mexican Americans and Pima Indians[7,22]. The mean BMI of Japanese diabetics is 23–25 according to various epidemiological studies, which is lower than that in other ethnic groups[4,23]. It has also been reported that the insulinogenic index in IGT is greater than that in NGT in Caucasians, in whom insulin secretion peaks at 121 mg/dL of plasma glucose[24] compared with 100 mg/dL in Japanese[25]. The diversities in these groups suggest the importance of characterizing both insulin secretion and sensitivity in the different stages of glucose intolerance. In the present study, for the prevention of type 2 diabetes and the selection of suitable treatments, we analyzed $IFG_{100-109}$, which has not been addressed with regard to insulin secretory capacity and insulin sensitivity. The present results in Japanese subjects with $IFG_{100-109}$ show that lesser reserve capacity of insulin secretion rather than greater insulin resistance is involved in deteriorating 2-hPG and AUC-G.

We have found that deterioration of both insulin secretory capacity and insulin sensitivity has already begun in $IFG_{100-109}$/NGT. In addition, early-phase insulin secretion plays an important role in the deterioration of postchallenge glucose levels among Japanese subjects with slightly impaired fasting glucose of $IFG_{100-109}$, whereas insulin sensitivity plays a more limited role. Over 40% of $IFG_{100-109}$ subjects were classified into IGT or diabetes according to 2-hPG during OGTT. Therefore, Japanese $IFG_{100-109}$ subjects classified into normal glucose tolerance according to the WHO criteria and the JDS criteria are recommended to be screened by OGTT to identify whether they are NGT, IGT or diabetes.

ACKNOWLEDGEMENTS

This research was supported by the Leading Project of Biosimulation, Kobe Translational Research Cluster and the Knowledge Cluster Initiative from the Ministry of Education, Culture, Sports, Science and Technology, Japan, Research grant from Japan Diabetes Society and Manpei Suzuki Diabetes Foundation. The authors do not have any financial support or relationships that pose a conflict of interest. We thank Use Techno Corporation, Ono Pharmaceutical Co. Ltd., Abbott Japan Co. Ltd. and Dainippon Sumitomo Pharma Co. Ltd. for their help in this study.

REFERENCES

1. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15: 539–553.
2. The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Invest* 2010; 1: 212–228.
3. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Follow-up on the diagnosis of diabetes mellitus. *Diabetes Care* 2003; 26: 3160–3167.
4. Fukushima M, Usami M, Ikeda Y, *et al.* Insulin secretion and insulin sensitivity at different stages of glucose tolerance: a cross-sectional study of Japanese type 2 diabetes. *Metabolism* 2004; 53: 831–835.
5. Mitsui R, Fukushima M, Nishi Y, *et al.* Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men. *Metabolism* 2006; 55: 53–58.
6. Haffner SM, D'Agostino R, Saad MF, *et al.* Increased insulin resistance and insulin secretion in nondiabetic African-Americans and Hispanics compared with non-Hispanic whites. The Insulin Resistance Atherosclerosis Study. *Diabetes* 1995; 45: 742–748.
7. Haffner SM, Stern MP, Hazuda HP, *et al.* Increased insulin concentrations in nondiabetic offspring of diabetic parents. *N Engl J Med* 1998; 319: 1297–1301.
8. National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 1979; 28: 1039–1057.
9. Suzuki H, Fukushima M, Usami M, *et al.* Factors responsible for development from normal glucose tolerance to isolated post challenge hyperglycemia. *Diabetes Care* 2003; 26: 1211–1215.
10. Taniguchi A, Fukushima M, Sakai M, *et al.* Remnant-like particle cholesterol, triglycerides, and insulin resistance in nonobese Japanese type 2 diabetic patients. *Diabetes Care* 2000; 23: 1766–1769.
11. Harada N, Fukushima M, Toyoda K, *et al.* Factors responsible for elevation of 1-h postchallenge plasma glucose levels in Japanese men. *Diabetes Res Clin Pract* 2008; 81: 284–289.
12. Abdul-Ghani MA, Jenkinson CP, Richardson DK, *et al.* Insulin secretion and action in subjects with impaired fasting glucose and impaired glucose tolerance: results from the Veterans Administration Genetic Epidemiology Study. *Diabetes* 2006; 55: 1430–1435.
13. Seltzer HS, Allen EW, Herron AL Jr, *et al.* Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes. *J Clin Invest* 1967; 46: 323–335.
14. Matsuda M, Defronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999; 22: 1462–1470.
15. Fukushima M, Suzuki H, Seino Y. Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. *Diabetes Res Clin Pract* 2004; 66: S37–S43.
16. Sato KK, Nakamura Y, Hayashi T, *et al.* Combined measurement of fasting plasma glucose and A1c is effective for the prediction of Type 2 diabetes. *Diabetes Care* 2009; 32: 644–646.
17. Bonora E, Kiechl S, Willeit J, *et al.* Population-based incidence rates and risk factors for type 2 diabetes in white individuals: the Bruneck study. *Diabetes* 2004; 53: 1782–1789.

18. Meigs JB, Muller DC, Nathan DM, *et al.* The natural history of progression from normal glucose tolerance to type 2 diabetes in the Baltimore Longitudinal Study of Aging. *Diabetes* 2003; 52: 1475–1484.
19. Eguchi H, Kato T, Tominaga M. Significance of IGT and impaired fasting glucose on the risk of development of diabetes. The Funagata study. *Diabetes (Japan)* 1998; 41: A107–A109.
20. Faerch K, Borch-Johnson K, Holsy JJ, *et al.* Pathophysiology and aetiology of impaired fasting glycaemia and impaired glucose tolerance: does it matter for prevention and treatment of type 2 diabetes? *Diabetologia* 2009; 52: 1714–1723.
21. Nishi Y, Fukushima M, Mitsui R, *et al.* Insulin secretion and insulin sensitivity in Japanese subjects with impaired fasting glucose and isolated fasting hyperglycemia. *Diabetes Res Clin Pract* 2005; 70: 46–52.
22. Saad MF, Knowler WC, Pettitt DJ, *et al.* A two-step model for development of non–insulin-dependent diabetes. *Am J Med* 1991; 90: 229–235.
23. Tripathy D, Carlsson M, Almgren P, *et al.* Insulin secretion and insulin sensitivity in relation to glucose tolerance: lessons from the Botnia Study. *Diabetes* 2000; 49: 975–980.
24. DeFronzo RA. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 1998; 37: 667–687.
25. Seino Y, Ikeda M, Yawata M, *et al.* The insulinogenic index in secondary diabetes. *Horm Metab Res* 1975; 7: 107–115.

Effects of glucose and meal ingestion on incretin secretion in Japanese subjects with normal glucose tolerance

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ABSTRACT

Aims/Introduction: Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the major incretins; their secretion after various nutrient loads are well-evaluated in Caucasians. However, little is known of the relationship between incretin secretion and differing nutritional loading in Japanese subjects. In the present study, we evaluated GIP and GLP-1 secretion in Japanese subjects with normal glucose tolerance (NGT) after glucose loading (75 g glucose and 17 g glucose) and meal ingestion.

Materials and Methods: A total of 10 Japanese NGT subjects participated in 75 g oral glucose tolerance test (OGTT), 17 g OGTT and meal tolerance test (MTT). Plasma glucose (PG), serum insulin (IRI), serum C-peptide (CPR), plasma total GIP, and plasma total GLP-1 levels during OGTT and MTT were determined.

Results: Area under the curve (AUC)-GIP was increased in proportion to the amount of glucose, and was highest in MTT, showing that GIP secretion is also stimulated by nutrients other than glucose, such as lipid. In contrast, although the larger glucose load tended to induce a larger GLP-1 release, AUC-GLP-1 was not significantly different among the three loading tests (75 g OGTT, 17 g OGTT, MTT) irrespective of the kind or amount of nutrition load.

Conclusions: Our results suggest that nutritional composition might have a greater effect on GIP secretion than that on GLP-1 secretion in Japanese NGT subjects. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00143.x, 2012)

KEY WORDS: Incretin, Meal tolerance test, Oral glucose tolerance test

INTRODUCTION

Oral glucose administration leads to greater insulin release from pancreatic islets than that by intravenous glucose loading yielding 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 on the surface of pancreatic β-cells to stimulate insulin secretion by increasing the intracellular adenosine 3′,5′-monophosphate concentration[2].

The incretin effect has been shown to be reduced in type 2 diabetic patients compared with that in normal glucose tolerance (NGT) subjects in previous studies[3,4], suggesting that a reduced incretin effect might be associated with hyperglycemia after food intake and glucose loading in type 2 diabetes. Plasma GLP-1 concentrations in type 2 diabetic patients have been reported to be reduced after meal ingestion and glucose loading[4,5]. However, in other studies, it was reported that GLP-1 concentrations did not differ in NGT and type 2 diabetic patients[6–8]. When intravenous infusion of GIP or GLP-1 was carried out in type 2 diabetic patients, GLP-1 potentiated insulin secretion from pancreatic β-cells, but GIP did not, showing that the GIP receptor (GIPR) signal is reduced in β-cells in type 2 diabetes[9]. In contrast, the GIPR signal plays an important role in maintaining blood glucose levels in the non-diabetic obese state[10,11]. Indeed, GIP concentrations are reported to be increased in obese rodent models and obese Caucasian subjects compared with those in lean rodents and lean Caucasian subjects, respectively[12–14]. In addition, we have previously shown hypersensitivity of GIPR to GIP in β-cells of high fat-induced obese mice[11]. In summary, evaluation of incretin secretion and the incretin effect in subjects with various levels of glucose tolerance is important to determine the contribution of incretin deficiency in progression from NGT to type 2 diabetes.

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity[15–17]. In Caucasians,

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Received 24 March 2011; revised 25 April 2011; accepted 16 May 2011

insulin resistance is thought to play a critical role in the pathogenesis of type 2 diabetes. In contrast, insulin sensitivity in Asian subjects has been shown to be higher than that in Mexican Americans and Caucasians in previous reports[18,19], which is partly because of the fact that Asians, including Japanese, are generally less obese. Thus, insulin secretion rather than insulin sensitivity is considered to be the more important factor in progression from NGT to diabetes in Japanese subjects[20]. Indeed, we have reported that early-phase insulin secretion is considerably decreased even in Japanese NGT subjects with 1-h plasma glucose levels higher than 10 mmol/L during an oral glucose tolerance test (OGTT)[21].

A recent study showed that, in both Caucasian NGT subjects and Caucasian type 2 diabetic patients, a meal tolerance test (MTT) elicited a significantly greater response of GIP levels than that elicited by OGTT, whereas GLP-1 levels were not different between OGTT and MTT[6]. In a previous study comparing the incretin secretion measured after different amounts of glucose load in healthy Caucasian subjects and type 2 diabetic Caucasian patients, GLP-1 and GIP were dose-dependently increased[22]. Plasma GLP-1 and GIP levels after glucose load or meal ingestion have been evaluated mainly in Caucasian subjects. In Japanese subjects, there has not been thorough elucidation, and little is known about the relationship between incretin secretion, and the kind and amount of nutrition load.

In the present study, we investigated incretin levels in association with the amount of glucose load and meal ingestion by measuring plasma GLP-1 and GIP levels after administration of 17 or 75 g glucose or mixed meal in Japanese NGT subjects.

MATERIALS AND METHODS

Subjects

A total of 10 healthy Japanese volunteers (eight male and two female) were recruited into the present study. 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 measurement of liver and kidney function, HbA_{1c} (National Glycohemoglobin Standardization Program), triglycerides (TG), total cholesterol and high-density lipoprotein (HDL)-cholesterol levels were drawn after an overnight fast. All subjects received 75 g OGTT, 17 g (approximately a quarter of 75 g) OGTT and a MTT. The interval between tests was 2–4 weeks. The total caloric content of the test meal was 450 kcal (carbohydrates 57.8 g, protein 17.2 g, fat 16.6 g). After the subjects fasted overnight for 10–16 h, OGTT or MTT was carried out according to the National Diabetes Data Group recommendations[23]. NGT was diagnosed according to World Health Organization (WHO) criteria[24].

Blood samples were collected at 0, 30, 60, 120 and 180 min after glucose loadings or meal ingestion 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. Blood was distributed into chilled tubes containing ethylenediaminetetraacetic acid and aprotinin (500 kIU/mL blood, Trasylol; SRL Inc., Tokyo, Japan) for analyses of GLP-1 and GIP. Plasma glucose (PG), serum insulin (IRI), serum C-peptide (CPR), plasma total GIP and plasma total GLP-1 were measured at the indicated times. The PG levels were measured by the glucose oxidase method. Serum IRI and CPR levels were measured by enzyme-linked immunosorbent assay. Total GIP and total GLP-1 levels were measured using a human GIP ELISA kit (Linco Research, St Charles, MO, USA) and human GLP-1 ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA), respectively, as previously described[25].

Calculations and Statistical Analysis

The area under the curve of PG (AUC-PG), IRI (AUC-IRI), CPR (AUC-CPR), total GIP (AUC-GIP) and total GLP-1 (AUC-GLP-1) were calculated by the trapezoidal rule. Statistical analyses were carried out using ANOVA and unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant. Data are presented as mean ± standard error (SE).

RESULTS

The profiles of the subjects are shown in Table 1. Mean age was 32.2 ± 2.0 years and mean body mass index was 22.4 ± 0.8 kg/m^2. Insulinogenic index, homeostasis model assessment (HOMA)-β and HOMA-insulin resistance were 0.59 ± 0.10, 76.50 ± 12.60, 1.10 ± 0.19, respectively. No subjects had liver or kidney dysfunction. HbA_{1c}, PG, TG, total cholesterol and HDL-cholesterol levels were within normal limits in the fasting state.

The profiles of PG, IRI and CPR in 75 g OGTT, 17 g OGTT and MTT are shown in Figure 1. Judging by the results of 75 g OGTT, all the subjects were diagnosed with NGT according to WHO criteria with fasting plasma glucose and 2 h glucose levels below 6.1 and 7.8 mmol/L, respectively. Fasting concentrations of PG, IRI and CPR were not different among the two OGTT and

Table 1 | Clinical characteristics of the subjects

n (Male/female)	10 (8/2)
Age (years)	32.2 ± 2.0
BMI (kg/m^2)	22.4 ± 0.8
Fasting plasma glucose (mmol/L)	4.9 ± 0.2
HbA_{1c} (%)	5.3 ± 0.1
Triglycerides (mg/dL)	79.4 ± 10.5
Total cholesterol (mg/dL)	169.2 ± 6.1
HDL-cholesterol (mg/dL)	61.5 ± 5.3
LDL-cholesterol (mg/dL)	93.0 ± 9.2

Data represent the mean ± SD. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

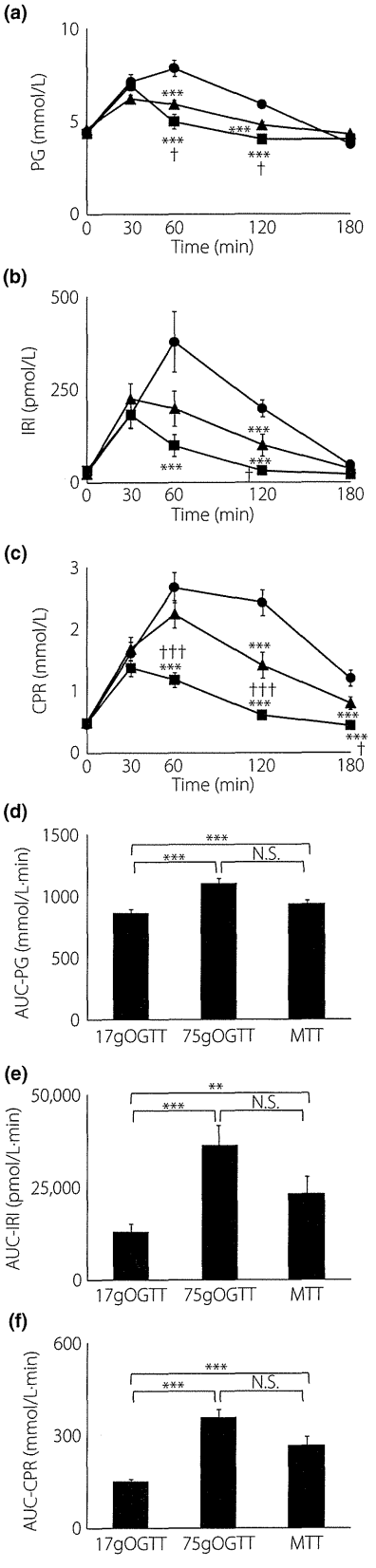

Figure 1 | Concentrations of (a) plasma glucose (PG), (b) serum insulin (IRI) and (c) serum C-peptide (CPR) during the 75 g oral glucose tolerance test (OGTT; closed circle), 17 g OGTT (closed square) and meal tolerance test (MTT; closed triangle) in 10 Japanese subjects. Asterisks indicate significant differences vs 75 g OGTT at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); daggers indicate significant differences vs MTT at individual time-points (†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$). (d) Area under the curve (AUC)-PG, (e) AUC-IRI, (f) AUC-CPR were calculated by the trapezoidal rule. Asterisks indicate significant differences at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Statistical analyses were carried out using ANOVA and unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant. Data are presented as mean ± standard error. N.S., not significant.

MTT. In OGTT studies, AUC-PG, AUC-IRI and AUC-CPR measured by the 75 g OGTT were significantly larger than those measured by the 17 g OGTT (Figure 1d–f). At 30 min after glucose ingestion, the levels of PG, IRI and CPR in the 75 g OGTT and those in the 17 g OGTT were not significantly different. Between MTT and the two OGTT, AUC-PG, AUC-IRI and AUC-CPR in MTT were significantly higher than those in the 17 g OGTT. AUC-PG, AUC-IRI and AUC-CPR in the 75 g OGTT and in MTT were not significantly different.

In the 17 g OGTT, the total GLP-1 level peaked at 30 min and rapidly decreased to the baseline at 60 min after the glucose load. The total GLP-1 level peaked at 30 min after the meal load and was sustained for up to 180 min. In the 75 g OGTT, the GLP-1 level peaked at 60 min and gradually decreased with time, but the level was still higher than baseline even at 180 min. The level of total GLP-1 at 60 min after the 75 g glucose load was significantly higher than that after the 17 g glucose load (Figure 2a). Although a larger glucose load tended to induce a larger GLP-1 release, total AUC-GLP-1 measured by the 75 g OGTT, 17 g OGTT and MTT were not significantly different (Figure 2b).

The baseline levels of GIP were approximately 10 pmol/L. The GIP level rapidly increased, peaked at 30 min after the meal load and gradually decreased with time, but the level was still higher than baseline even at 180 min. In the 75 g OGTT, the GIP level significantly increased at 30 min after the glucose load, peaked at 120 min and were maintained up to 180 min. In the 17 g OGTT, the total GIP level peaked at 30 min after glucose load and gradually decreased to baseline at 180 min. At 30 min after ingestion, total GIP levels in the 75 g OGTT and those in the 17 g OGTT were not significantly different (Figure 3a).

AUC-GIP was significantly higher in the 75 g OGTT than that in the 17 g OGTT. Unlike GLP-1, the peak levels of GIP and the AUC-GIP measured in the MTT were significantly higher than those measured in the 75 g OGTT and 17 g OGTT (Figure 3b).

DISCUSSION

In the present study, incretin levels were estimated after glucose loading or meal ingestion in Japanese NGT subjects.

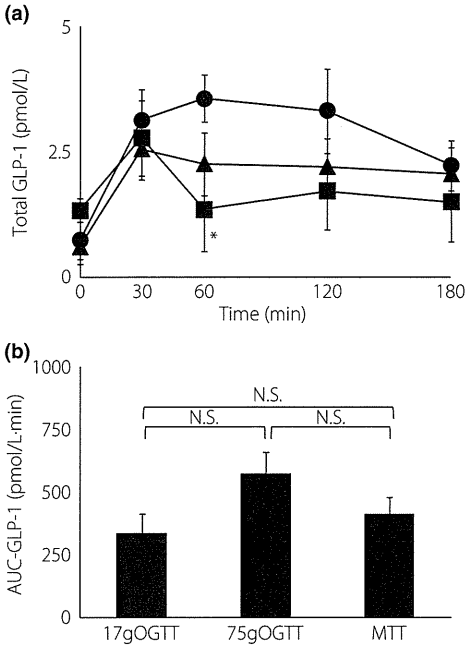

Figure 2 | (a) Concentrations of total glucagon-like peptide-1 (GLP-1) during the 75 g oral glucose tolerance test (OGTT; closed circle), 17 g OGTT(closed square) and meal tolerance test (MTT; closed triangle) in 10 Japanese subjects. Asterisks indicate significant differences vs 75 g OGTT at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); daggers indicate significant differences vs MTT at individual time-points (†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$). (b) Area under the curve (AUC)-GLP-1 was calculated by the trapezoidal rule. Asterisks indicate significant differences at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Statistical analyses were carried out using ANOVA and unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant. Data are presented as mean ± standard error. N.S., not significant.

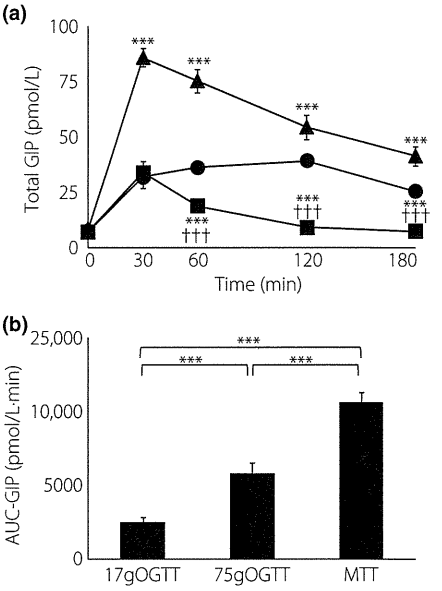

Figure 3 | (a) Concentrations of total gastric inhibitory polypeptide (GIP) during 75 g oral glucose tolerance test (OGTT; closed circle), 17 g OGTT (closed square) and meal tolerance test (MTT; closed triangle) in 10 Japanese subjects. Asterisks indicate significant differences vs 75 g OGTT at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); daggers indicate significant differences vs MTT at individual time-points (†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$). (b) Area under the curve (AUC)-GIP was calculated by the trapezoidal rule. Asterisks indicate significant differences at individual time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Statistical analyses were carried out using ANOVA and unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant. Data are presented as mean ± standard error.

Between the OGTT studies, AUC-PG, AUC-IRI and AUC-CPR in the 75 g OGTT were larger than those in the 17 g OGTT. Regarding incretins, AUC-GIP was significantly larger in the 75 g OGTT than in the 17 g OGTT. In contrast, AUC-GLP-1 was not significantly different between the 75 g OGTT and the 17 g OGTT. Previous studies showed that a larger amount of oral glucose load elicited more GIP and GLP-1 secretion[1,22], whereas a recent study also reported that the secretory response of GIP was more sensitive than that of GLP-1 to changes in intestinal carbohydrate content[26]. The present study also showed that while GLP-1 level was not increased, GIP level was increased dose-dependently in response to glucose load, showing higher sensitivity of GIP to changes of administered nutrient dose.

Between the 75 g OGTT and MTT studies, AUC-PG, AUC-IRI and AUC-CPR were not significantly different. AUC-GIP was significantly larger in MTT than that in the 75 g OGTT. In contrast, there was no significant difference in AUC-GLP-1 among the MTT and the two OGTT. By comparing the results of the three loading tests (75 g OGTT, 17 g OGTT, MTT), we speculate that AUC-GIP is more susceptible to the contents of each loading test than AUC-GLP-1 is. Vollmer *et al.*[6] reported that GIP responses were significantly higher in MTT than in OGTT, whereas GLP-1 levels were similar in both tests in Caucasian NGT, IGT and type 2 diabetic subjects. Because the mixed meal contains not only carbohydrates but also fat, which has been reported to stimulate GIP secretion[27–29], it is likely that the increased GIP concentrations after MTT were largely as a result of the fat content, which might have had no additional impact on GLP-1 secretion.

There are two previous reports that evaluate the incretin levels in both OGTT and MTT in Japanese NGT subjects[8,30]. However, they compared the incretin levels in 75 g glucose or meal load between NGT and type 2 diabetic subjects, but did not compare the incretin levels between 75 g glucose and meal load directly. The present study directly compared the incretin levels in the two OGTT and MTT. Our data clearly show that GIP responses were significantly higher in MTT than those in the two OGTT, whereas GLP-1 levels were not different between the two OGTT and MTT in Japanese NGT subjects.

According to the study by Yabe *et al.*[8], AUC-GIP is similar between the OGTT and MTT group in Japanese control subjects. It should be noted that the difference between GIP secretion after meal load and that after glucose load was far greater in the present study than that in the study by Yabe *et al.* The total caloric content of the test meal used in their study was 480 kcal (carbohydrates 58.4%, protein 20.8%, fat 20.8%) and that in the present study was 450 kcal (carbohydrates 51.4%, protein 15.3%, fat 33.3%). Therefore, it is possible that the higher amount of contained fat in the test meal used in the present study led to the greater response of GIP secretion in the MTT.

Fasting and peak total GLP-1 concentrations in the present study were approximately 1 pmol/L and 3.5 pmol/L, respectively, and seemed to be lower than those in some published results[8,31]. However, in other reports, total GLP-1 levels after glucose and meal load were not very different from those in the present study. Rijkelijkhuizen *et al.*[32] measured the total GLP-1 concentration with radioimmunoassay, and in their results, the fasting and peak total GLP-1 concentrations in the MTT were approximately 1 pmol/L and 4.5 pmol/L, respectively. In addition, Villareal *et al.*[33] evaluated total GLP-1 concentrations by the same method that we used in the present study, and reported that the fasting and peak total GLP-1 concentrations in OGTT were approximately 1.5 and 6 pmol/L, respectively. Judging by the data in these reports, it is not necessarily the case that total GLP-1 concentrations were extremely low in the present study.

There are some reports showing that GLP-1 secretion is dependent on meal size, especially on carbohydrate and glucose loads. Schirra *et al.*[34] reported that GLP-1 plasma levels rose from basal levels to fourfold after 50 g glucose ingestion and to eightfold after 100 g glucose ingestion. Rijkelijkhuizen *et al.*[32] showed that GLP-1 secretion is increased by the amount of carbohydrate (75 and 109 g) and not by the quantity of the meal. In the present study, however, AUC-GLP-1 was not significantly different among the three loading tests (75 g OGTT, 17 g OGTT, MTT) irrespective of kinds or amounts of nutrition load, although larger glucose load tended to induce a larger GLP-1 release. The most notable difference between the previous studies and the present study was the amount of glucose load. We compared GLP-1 secretion after administration of 17 g glucose, 75 g glucose and 57.8 g of carbohydrate contained in the meal that we used. The amount of glucose and carbohydrate load in the present study were relatively lower than those in the previous studies. It is possible that evaluation of GLP-1 secretion after larger glucose loads could be more appropriate to show the glucose dependency of GLP-1 secretion.

It is also noteworthy that the levels of PG, IRI, CPR, GIP and GLP-1 at 30 min after the 75 g OGTT and 17 g OGTT were similar to each other. In addition, the levels of IRI, CPR and GLP-1 at 30 min after MTT and the two OGTT were not significantly different. By contrast, the GIP level at 30 min after MTT was much higher than those after the 17 g OGTT and 75 g OGTT. Given the similar plasma glucose levels at 30 min after the 17 g OGTT and 75 g OGTT, it is likely that under physiological conditions, the rate at which ingested glucose emptied into the duodenum is regulated finely enough to prevent an abrupt increase in plasma glucose levels irrespective of the amount of ingested glucose. Previous studies have shown that GLP-1 secretion after a test meal or oral glucose load is associated with the rate of gastric emptying, whereas GIP secretion seems to be dependent on nutrient absorption rather than on rate of gastric emptying[34]. Accordingly, a finely regulated rate of gastric emptying might account for the similar levels of GLP-1 at 30 min after MTT, 17 g OGTT and 75 g OGTT. In contrast, the level of total GIP at 30 min after the MTT was much higher than those after the two OGTT, probably because of the presence of fat in the duodenal lumen, as fat is a forcible stimulant of GIP, as discussed earlier.

The present results clearly show that the secretion of GIP and GLP-1 are regulated by different nutrient factors. On the basis of our data, it is also suggested that nutritional composition might have a greater effect on GIP secretion than on GLP-1 secretion in Japanese NGT subjects.

ACKNOWLEDGEMENTS

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

REFERENCES

1. Nauck MA, Homberger E, Siegel EG, *et al.* Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab* 1986; 63: 492–498.
2. Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormone: similarities and difference. *J Diabetes Invest* 2010; 1: 8–23.
3. Nauck M, Stockmann F, Ebert R, *et al.* Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 1986; 29: 46–52.
4. Muscelli E, Mari A, Casolaro A, *et al.* Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 2008; 57: 1340–1348.
5. Vilsboll T, Krarup T, Deacon CF, *et al.* Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. *Diabetes* 2001; 50: 609–613.
6. Vollmer K, Holst JJ, Baller B, *et al.* Predictors of incretin concentrations in subjects with normal, impaired, and diabetic glucose tolerance. *Diabetes* 2008; 57: 678–687.
7. Faerch K, Vaag A, Holst JJ, *et al.* Impaired fasting glycaemia vs impaired glucose tolerance: similar impairment of pancreatic alpha and beta cell function but differential roles of incretin hormones and insulin action. *Diabetologia* 2008; 51: 853–861.

8. Yabe D, Kuroe A, Lee S, *et al.* Little enhancement of meal-induced glucagon-like peptide 1 secretion in Japanese: comparison of type 2 diabetes patients and healthy controls. *J Diabetes Invest* 2010; 1: 56–59.
9. Nauck MA, Heimesaat MM, Orskov C, *et al.* Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 1993; 91: 301–307.
10. Miyawaki K, Yamada Y, Yano H, *et al.* Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci USA* 1999; 96: 14843–14847.
11. Harada N, Yamada Y, Tsukiyama K, *et al.* A novel GIP receptor splice variant influences GIP sensitivity of pancreatic beta-cells in obese mice. *Am J Physiol Endocrinol Metab* 2008; 294: E61–E68.
12. Miyawaki K, Yamada Y, Ban N, *et al.* Inhibition of gastric inhibitory polypeptide signaling prevents obesity. *Nat Med* 2002; 8: 738–742.
13. Flatt PR, Bailey CJ, Kwasowski P, *et al.* Abnormalities of GIP in spontaneous syndromes of obesity and diabetes in mice. *Diabetes* 1983; 32: 433–435.
14. Creutzfeldt W, Ebert R, Willms B, *et al.* Gastric inhibitory polypeptide (GIP) and insulin in obesity: increased response to stimulation and defective feedback control of serum levels. *Diabetologia* 1978; 14: 15–24.
15. Mitrakou A, Kelley D, Mokan M, *et al.* Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose tolerance. *N Engl J Med* 1992; 326: 22–29.
16. Haffner SM, Stern MP, Hazuda HP, *et al.* Increased insulin concentrations in nondiabetic offspring of diabetic parents. *N Engl J Med* 1988; 319: 1297–1301.
17. Saad MF, Knowler WC, Pettitt DJ, *et al.* A two-step model for development of non-insulin-dependent diabetes. *Am J Med* 1991; 90: 229–235.
18. Chiu KC, Chuang LM, Yoon C. Comparison of measured and estimated indices of insulin sensitivity and beta cell function: impact of ethnicity on insulin sensitivity and beta cell function in glucose-tolerant and normotensive subjects. *J Clin Endocrinol Metab* 2001; 86: 1620–1625.
19. Mandavilli A, Cyranoski D. Asia's big problem. *Nat Med* 2004; 10: 325–327.
20. Seino Y, Ikeda M, Yawata M, *et al.* The insulinogenic index in secondary diabetes. *Horm Metab Res* 1975; 7: 323–335.
21. Harada N, Fukushima M, Toyoda K, *et al.* Factors responsible for elevation of 1-h postchallenge plasma glucose levels in Japanese men. *Diabetes Res Clin Pract* 2008; 81: 284–289.
22. Bagger JI, Knop FK, Lund A, *et al.* Impaired regulation of the incretin effect in patients with type 2 diabetes. *J Clin Endocrinol Metab* 2011; 96: 737–745.
23. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. National Diabetes Data Group. *Diabetes* 1979; 28: 1039–1057.
24. Alberi KG, Zimmeret PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 54: 539–553.
25. Harada N, Hamasaki A, Yamane S, *et al.* Plasma GIP and GLP-1 levels after glucose loading are associated with different factors in Japanese subjects. *J Diabetes Invest* 2011; 2: 193–199.
26. Yoder SM, Yang Q, Kindel TL, *et al.* Differential responses of the incretin hormones GIP and GLP-1 to increasing doses of dietary carbohydrate but not dietary protein in lean rats. *Am J Physiol Gastrointest Liver Physiol* 2010; 299: G476–G485.
27. Brown JC, Dryburgh JR, Ross SA, *et al.* Identification and actions of gastric inhibitory polypeptide. *Recent Prog Horm Res* 1975; 31: 487–532.
28. Falko JM, Crockett SE, Cataland S, *et al.* Gastric inhibitory polypeptide (GIP) stimulated by fat ingestion in man. *J Clin Endocrinol Metab* 1975; 41: 260–265.
29. Pederson RA, Schubert HE, Brown JC. Gastric inhibitory polypeptide. Its physiologic release and insulinotropic action in the dog. *Diabetes* 1975; 24: 1050–1056.
30. Lee S, Yabe D, Nohtomi K, *et al.* Intact glucagon-like peptide-1 levels are not decreased in Japanese patients with type 2 diabetes. *Endocr J* 2010; 57: 119–126.
31. Kozawa J, Okita K, Imagawa A, *et al.* Similar incretin secretion in obese and non-obese Japanese subjects with type 2 diabetes. *Biochem Biophys Res Commun* 2010; 393: 410–413.
32. Rijkelijkhuizen JM, McQuarrie K, Girman CJ, *et al.* Effects of meal size and composition on incretin, alpha-cell, and beta-cell responses. *Metabolism* 2010; 59: 502–511.
33. Villareal DT, Robertson H, Bell GI, *et al.* TCF7L2 variant rs7903146 affects the risk of type 2 diabetes by modulating incretin action. *Diabetes* 2010; 59: 479–485.
34. Schirra J, Katschinski M, Weidmann C, *et al.* Gastric emptying and release of incretin hormones after glucose ingestion in humans. *J Clin Invest* 1996; 97: 92–103.