GlucaGon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted in response to ingestion of nutrients. In the circulation, GLP-1 and GIP are rapidly inactivated by dipeptidyl peptidase-IV (DPP-IV), which cleaves off two N-terminal amino acids [1].

Recently, Vilsbøll et al. reported that postprandial plasma levels of immunoreactive active glucagon-like peptide-1 (p-active GLP-1) and immunoreactive total GLP-1 (p-total GLP-1) in patients with type 2 diabetes [2]. We reexamined the effects of a test meal (TM) similar to the one used by Vilsbøll et al. [2] on postprandial levels of p-active GLP-1 in type 1 diabetic Japanese patients who used subcutaneous injections of bolus rapid-acting insulins. Here, we report findings that differed from those of Vilsbøll et al. [2].

Materials and Methods

Japanese patients with type 1 diabetes (6 acute, 2 fulminant, and 2 slowly progressive), who visited our clinics regularly (n=10, group 1) and control subjects with normal glucose tolerance (NGT) (n=15, group 2) were studied. Patients with type 1 diabetes but with hypoglycemia were excluded from this study. Type 1 diabetic patients and control subjects were matched by sex, age, and body mass index (BMI). Demographic characteristics of the participants are presented in Table 1. Diabetic patients were diagnosed using the World Health Organization criteria of the American Diabetes Association. The study was approved by the Institutional Research Committee of the Nagaoka Red Cross Hospital and the Department of Health and Nutrition, University of Niigata Prefecture. All patients provided written informed consent for participation in the study.
Blood samples were collected in ice-cooled tubes from the inserted cannula immediately before, and 30 and 60 min after ingestion of TM. Samples were separated by centrifugation at 4°C for later determination of plasma glucose (PG), serum immunoreactive insulin (s-IRI), s-CPR and p-active GLP-1 levels. The sample collected was also used to measure HbA1c levels.

Blood samples for active GLP-1 and glucose were collected in ice-cooled vacutainers containing EDTA with 10 μL DPP-IV inhibitor (diprotin) per mL of blood [4] and in vacutainers containing NaF, respectively.

Increment levels of PG, s-CPR, s-IRI and p-active GLP-1 after ingestion of TM were calculated from the integrated areas under curves (AUC) from 0 to 60 min based on the method described by Vilsbøll et al. [2]. HbA1c was measured using Japanese JCCLS CRM-004 as a standard by high-performance liquid chromatography (Toso, Tokyo, Japan) [5]. The value was

### Table 1 Demographic and descriptive characteristics of the study participants

<table>
<thead>
<tr>
<th></th>
<th>Type 1 diabetic patients</th>
<th>Control subjects</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>5/5</td>
<td>10/5</td>
<td>0.442</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>51 ± 5</td>
<td>49 ± 2</td>
<td>0.734</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.3 ± 0.7</td>
<td>21.1 ± 0.4</td>
<td>0.179</td>
</tr>
<tr>
<td>HbA1c (%) (NGSP)</td>
<td>7.3 ± 0.2</td>
<td>5.3 ± 0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>164 ± 20</td>
<td>91 ± 1.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum immunoreactive insulin (µU/mL)</td>
<td>4.9 ± 0.7</td>
<td>3.9 ± 0.7</td>
<td>0.314</td>
</tr>
<tr>
<td>Serum immunoreactive C-peptide (ng/mL)</td>
<td>0.2 ± 0.01</td>
<td>1.3 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma immunoreactive active GLP-1 (pmol/L)</td>
<td>1.7 ± 0.3</td>
<td>2.8 ± 0.6</td>
<td>0.130</td>
</tr>
<tr>
<td>Duration of therapy (year)</td>
<td>19.0 ± 3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDI (lispro or aspart + glargine or detemir)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSII (lispro)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic complications</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are expressed as means ± SEM. All samples were collected in the fasting. MDI: multiple daily insulin injection therapy; CSII: continuous subcutaneous insulin therapy. Differences between the means 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.

Control subjects were recruited from persons with normal glucose tolerance (NGT) for a 75-g oral glucose tolerance test (OGTT) [3]. None of the participants had a history of gastrointestinal disease, anemia, micro- and macro-vascular disturbances, or impaired liver function, and none were receiving any other medications.

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

After 10-hr overnight fast, subjects were studied in a sitting position at 9:00 a.m. with one cannula inserted into the cubital vein for blood sampling based on the method of Vilsbøll et al. [2]. The TM (560 kcal) comprised 23% fat, 60% carbohydrate, and 17% protein. Patients with type 1 diabetes received subcutaneous injections of long-acting insulin analogues before going to sleep on the previous day as basal; and received subcutaneous injections of either rapid-acting insulin analogues as bolus in MDI or those of rapid-acting insulin analogues as basal and bolus in CSII, before ingestion of TM on the day of study.

Blood samples were collected in ice-cooled tubes from the inserted cannula immediately before, and 30 and 60 min after ingestion of TM. Samples were separated by centrifugation at 4°C for later determination of plasma glucose (PG), serum immunoreactive insulin (s-IRI), s-CPR and p-active GLP-1 levels. The sample collected was also used to measure HbA1c levels.

Blood samples for active GLP-1 and glucose were collected in ice-cooled vacutainers containing EDTA with 10 μL DPP-IV inhibitor (diprotin) per mL of blood [4] and in vacutainers containing NaF, respectively.

Increment levels of PG, s-CPR, s-IRI and p-active GLP-1 after ingestion of TM were calculated from the integrated areas under curves (AUC) from 0 to 60 min based on the method described by Vilsbøll et al. [2]. HbA1c was measured using Japanese JCCLS CRM-004 as a standard by high-performance liquid chromatography (Toso, Tokyo, Japan) [5]. The value was
expressed as a NGSP (National Glycohemoglobin Standardization Program) equivalence; i.e., NGSP equivalent value (%) = JDS (Japan Diabetes Society) value (%) + 0.4 %.

PG was measured by using the oxidase method (N-assay Glu-UL, Nittobo Medical Co., Tokyo, Japan).

ST AIA-PACK IRI and C-peptide kits (Tosho Co., Tokyo, Japan) were used to measure s-IRI and s-CPR, respectively. The limit of detection for s-IRI is 1.1 μU/mL and that for s-CPR is 0.2 ng/mL. The intra- and inter-assay coefficients of variation were both <5%. IRI assay showed 100%, 75% and 2%, and 0% cross-reactivity with human-, ovine- and pro-insulin and human CPR, respectively. CPR assay showed 100%, 69% and 0% cross-reactivity with human CPR, pro-insulin and human-insulin, respectively.

P-active GLP-1 was measured by two-site sandwich immunoassay using a commercially available active GLP-1 Linco kit (Linco Research, St. Charles, MO, USA) [4]; 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 is <2.0 pmol/L. The intra- and inter-assay coefficients of variation are both <13% [4].

Results are expressed as means ± SEM. Difference between means of basal state, insulinogenic index or AUC in groups was 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 the response was affected by ingestion of TM in groups. Following the one-way test or the two-way test, a Bonferroni’s test was used as a post hoc test to compare variables in groups. Two-tailed p-values <0.05 were considered as statistically significant.

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

Results
There was no significant difference in mean of sex, age or BMI between groups (Table 1). Means of HbA1c and basal PG were significantly higher in group 1 than in group 2 (Table 1), whereas mean of basal s-CPR was significantly lower in group 1 than in group 2. There was no significant difference in mean of basal s-IRI or p-active GLP-1 levels between groups (Table 1). In group 1, the mean duration of treatment for disease was 19 years and no clinically diabetic complications were seen (Table 1).

Means of postprandial PG and s-IRI levels at each time after ingestion of TM were significantly higher in group 1 than in group 2, whereas means of postprandial s-CPR and p-active GLP-1 levels at each time after ingestion of TM were significantly lower in group 1 than in group 2, although the peak levels of both s-IRI and p-active GLP-1 in two groups were observed at 30 min after ingestion of TM (Fig. 1). There was no significant change in postprandial s-CPR levels at each time after ingestion of TM in group 1.

The means AUC of PG and s-IRI were significantly higher in group 1 (576 ± 70 mg min/dL and 131 ± 19 µU min/mL, respectively) than in group 2 (308 ± 10 mg min/dL and 51 ± 3.9 µU min/mL, respectively) (p<0.001). The means AUC of s-CPR and p-active GLP-1 were significantly lower in group 1 (0.7 ± 0.1 ng min/mL and 7.3 ± 0.6 pmol min/L, respectively) than in group 2 (8.8 ± 0.5 ng min/mL and 19.2 ± 2.7 pmol min/L, respectively) (p<0.001).

Discussion
The patients had type 1 diabetes and had no diabetic complications. As they visited our clinics regularly, the mean duration of treatment for disease increased to more than 10 years and was longer than that in patients by Vilsbøll et al. Like the subjects in study of Vilsbøll et al., our patients lacked endogenous insulin secretion based on s-CPR levels because of β cell failure [2, 6].

We found that, although there was no significant difference in mean of fasting p-active GLP-1 levels between the patients and the control subjects, a lower response of postprandial p-active GLP-1 levels following ingestion of TM was observed in the patients than in the control subjects. Vilsbøll et al. reported that responses of postprandial p-active GLP-1 and p-total GLP-1 levels following ingestion of TM were not lower in type 1 diabetic patients who did not take bolus insulin than in control subjects with NGT [2].

In the present study, the methods were similar to those used by Vilsbøll et al. [2]. However, there were some differences in the two studies. First, the differences between two studies may be due to differences in the assays used to measure p-active GLP-1. We used the assay developed by Linco Co., whereas Vilsbøll et al. used an assay developed by their group [2].
Fig. 1 Change in blood concentrations of glucose (A), immunoreactive insulin (IRI) (B), immunoreactive C-peptide (CPR) (C), and immunoreactive active GLP-1 (D) before, 30 and 60 min after ingestion of a breakfast test meal (560 kcal) in Japanese patients with type 1 diabetes (●, n=10) or control subjects with normal glucose tolerance (○, n=15) following 10 hr of overnight fasting. The mean BMI was not significantly different between groups. The data are expressed as means ± SEM. The patients were treated with multiple daily injections or CSII 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. group with control subjects).
assay used in this study may have higher variation and lower specificity than the assay developed by Vilsbøll et al. (personal communication for T. Vilsbøll in March, 2011). Nevertheless, the assay developed by Vilsbøll et al. is similar to the Linco kit assay because of 100% cross-reactivity with active GLP-1 and no cross-reactivity with other forms of GLP-1 (1–37, 8–37 and 9–37) [2, 4]. But, the means of basal (around 5 pmol/L) and postprandial (around 29 pmol min/L) values with AUC (0–60 min) in p-active GLP-1 after ingestion of TM in the patients by Vilsbøll et al. were about 2 and 4 times higher than those in the present study, respectively. The values, however, were about half of those in the control, although they reported that the increment of response in the patients was normal in comparison with control subjects [2]. The higher levels of p-active GLP-1 by Vilsbøll et al. compared with those by our study indicate that the method used by Vilsbøll et al. may measure some inactive GLP-1, although they reported that the assay measures only active GLP-1 [2]. Moreover, Lugari et al. [7] found that, following ingestion of a small TM (230 kcal), p-active GLP-1 levels in patients with type 1 diabetes were as low as those observed in our patients. However, the patients in the study of Lugari et al. had not received bolus insulin during the study, and their samples were drawn into tubes containing EDTA and aprotinin without a DPP-IV inhibitor [7]. Our unpublished observation indicates that p-active GLP-1 levels in samples of healthy subjects collected without DPP-IV inhibitor could not be measured by using the Linco assay kit. Therefore, the p-active GLP-1 levels in the samples without DPP-IV inhibitor might be low even though patients did not use bolus insulin. Increased p-active GLP-1 levels in control subjects with NGT after TM may be due to inclusion of other forms of GLP-1 in the measurements, although the assay by Lugari et al. cross-reacted with 100% of active GLP-1, but did not cross-react with other forms of GLP-1 (1-37), GLP-1 (7-37) and GLP-2 [7]. In a preliminary study, we measured basal and postprandial p-active GLP-1 levels following consumption of the TM used in the current study in non-obese type 2 diabetic Japanese patients treated without insulin therapy, and we found that the mean of postprandial p-active GLP-1 levels is about 3 times increased after treatment with DPP-IV inhibitor sitagliptin [AUC (0–60 min); from 16.0±3.5 to 50.0±5.0 pmol min/L], although there was no significant difference in the means of basal and postprandial p-active GLP-1 levels between patients and control subjects with NGT before the treatment with sitagliptin. Therefore, measurement of p-active GLP-1 using the Linco assay kit may not be problematic in the clinical setting, as indicated by Vahl et al. [4].

Alternatively, the differences between our findings and those of Vilsbøll et al. may be due to glycemic state. Although the PG means of basal levels and postprandial levels after ingestion of TM were higher in our patients than in the control subjects, they were lower than those in patients studied by Vilsbøll et al., and the mean of HbA1c levels was also lower in our patients than in patients by the Vilsbøll et al. [2]. Hyperglycemia evaluated by HbA1c levels increases DPP-IV activity in patients with type 1 diabetes [8]. Moreover, serum DPP-IV activity is positively correlated to fasting plasma glucose and HbA1c levels in patients with type 2 diabetes [9]. Hence, the increased DPP-IV activity in the patients studied by Vilsbøll et al. should result in lower p-active GLP-1 levels relative to those in our patients, but the p-active GLP-1 levels were actually higher than those seen in our patients [2]. Furthermore, the p-active GLP-1 levels may increase when patients have hypoglycemia, as in patients with type 2 diabetes [9], but no patients with hypoglycemia participated in either study. Therefore, glycemic state may be not directly related to these differences between the studies.

Different subjects were examined in the two studies, and this factor may have caused the differences. We examined Japanese, while Vilsbøll et al. studied Europeans. The genetic factors that influence type 1 diabetes in the Japanese population are different from those in the European one [2]. Therefore, different genetic factors may have contributed to the differences in p-active GLP-1 levels, but there is no clear evidence to support this hypothesis.

Fourth, DPP-IV activity may contribute to the differences. Fasting s-IRI levels in our patients and the control subjects were not significantly different, but Vilsbøll et al. found that the fasting s-IRI levels were higher in their patients than in the control subjects. The patients in both studies lacked the endogenous insulin secretion and the levels of postprandial s-IRI after ingestion of TM observed by Vilsbøll et al. which were actually lower in patients than in control subjects [2], but the levels of postprandial s-IRI after ingestion of TM were significantly higher in our patients than in the control subjects. The s-IRI levels were estimated by different
immunoreactive methods in the two studies; therefore, it may not be appropriate to directly compare the findings, because of the fact that the s-IRI levels determined by the immunoreactive methods are influenced by the various kinds of insulin. Accordingly, although it is not easy to draw a conclusion, the differences may be due to the differences in the kind of insulin and doses, particularly bolus insulin versus non-bolus insulin. Our patients received bolus rapid-acting insulin analogues during the study; in contrast, the patients in the study by Vilsbøll et al. did not use bolus insulin at the start of the study. Although we did not measure p-total GLP-1 levels, Vilsbøll et al. reported that there was no statistical difference in mean of basal p-total GLP-1 levels between patients and control, but the mean of postprandial levels with AUC (around 105 pmol min/L) of p-total GLP-1 after ingestion of TM in patients was significantly higher than that (around 78 pmol min/L) in the control subjects [2]. Furthermore, Hare et al. reported that the mean of postprandial p-total GLP-1 levels after 50 g OGTT in type 1 diabetic patients who did not take bolus insulin throughout the study was also significantly higher than those in control subjects with NGT, although there was no significant difference in mean of basal p-total GLP-1 levels between the patients and the controls, based on measurements using the same assay for p-total GLP-1 [10]. Accordingly, the differences of basal and postprandial p-active GLP-1 levels may be due, in part, to the different activity of DPP-IV, which is affected by insulin. Insulin may increase the activity of DPP-IV. In vitro, DPP-IV activity in rat liver is increased by insulin-dependent phosphorylation [11]. Moreover, a recent report indicates that serum DPP-IV activity is elevated in type 1 diabetic patients who used bolus insulin [12]. Thus, the patients studied by Vilsbøll et al. may have increased basal DPP-IV activity by insulin and decreased postprandial DPP-IV activity owing to a lack of insulin, whereas the activity of postprandial DPP-IV after ingestion of TM in our patients may be more increased by an overdose of insulin that resulted from subcutaneous injections of bolus rapid-acting insulin.

To test the hypothesis, p-active GLP-1 levels with p-total GLP-1 and serum DPP-IV activity should be measured before and after ingestion of TM in the type 1 diabetic patients who did not use subcutaneous injection of bolus rapid-acting insulin analogues before the start of the study. However, this protocol was not used both because of humanitarian reasons and because the measurement of DPP-IV activity in serum is not accurately estimated as a function of DPP-IV activity, since serum DPP-IV is about 5% of all DPP-IVs in the human as reported by Ryskjaer et al. [9]. Therefore, the hypothesis remains to be resolved.

Fifth, the differences between the two studies may be explained by a negative-feedback control between insulin and active GLP-1 secretions as the report by Creutzfeldt et al. [13]. Matsuyama et al. also reported that insulin inhibits GLP secretion in patients with type 1 diabetes [14].

Finally, Shigeto et al. reported that low, but physiological concentration of GLP-1 has a role in stimulating insulin secretion independent of the cAMP-dependent protein kinase pathway in vitro [15]. As these cases had no clinical diabetic complications for a long time (about 19 years) from the initial therapies, the low response of postprandial p-active GLP-1 levels following ingestion of TM may have a physiologic function in other regions except the β cell. This issue also needs to be resolved in the future.

**Limitations of this study**

The ratios of nutrients in the TM used in this study were different from those used in Vilsbøll et al. [2]. Although the total calorie (560 kcal) in this study was similar to that (520 kcal) in Vilsbøll et al., the ratios of nutrients were 23% fat, 60% carbohydrate, and 17% protein in TM of our study, whereas they were 33% fat, 48% carbohydrate and 19% protein in TM of Vilsbøll et al. [2]. Thus, the ratios of carbohydrate and fat were higher and lower in this study than in Vilsbøll et al., respectively. Therefore, it is not be completely ruled out that there is a different response in p-active GLP levels to TM between the two groups because of the different ratios of nutrients in the TM used.

In conclusion, our results indicated that a decreased response of postprandial plasma active GLP-1 levels following ingestion of a TM was seen in type 1 diabetic Japanese patient who received subcutaneous injections of bolus rapid-acting insulin analogue.

**Acknowledgments**

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References