Basal glucagon hypersecretion and response to oral glucose load in prediabetes and mild type 2 diabetes

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Abstract. Dysregulation of glucagon secretion plays an important role in the pathogenesis of type 2 diabetes (T2DM). However, it hasn’t been elucidated involvement of glucagon dysregulation in pathophysiology of T2DM. Recently, a new glucagon sandwich enzyme-linked immunosorbent assay (ELISA) became available that can measure plasma glucagon level with higher accuracy and simpler procedure than the conventional RIA method. We performed OGTT for adult subjects aged 20–69 years to define normal glucose tolerance (NGT, n = 25), borderline glucose intolerance (defined as pre-diabetes mellitus: preDM, n = 15), or diabetes mellitus (DM, n = 13), and we measured glucagon levels with this new ELISA method at fasting and during OGTT. Plasma glucose, insulin, glucagon and active GLP-1 were also measured. This study took place in diabetes outpatient clinic in Kitasato University Hospital and an affiliated outpatient clinic. PreDM and DM exhibited higher fasting plasma glucagon levels than NGT (34.4 ± 4.6 and 44.1 ± 5.0 vs. 20.6 ± 3.6 pg/mL), and statistical significance was observed between NGT and DM (p < 0.05). There was significant correlation between fasting glucagon level and indexes of insulin sensitivity. During OGTT, glucagon levels were less suppressed in DM and preDM than in NGT, whereas no apparent relationship was observed between glucagon and GLP-1 secretion. Significant positive correlation was observed between glucagon levels during OGTT and fasting TG. In conclusion, subjects with mild T2DM exhibited fasting hyperglucagonemia and insufficient suppression to oral glucose load compared to NGT subjects.

Key words: Glucagon, Type 2 diabetes, Prediabetes, Oral glucose load, Hyperglucagonemia

THE PATHOPHYSIOLOGY of type 2 diabetes has long been focused on the mechanisms behind reduced insulin action such as insulin resistance and decreased insulin secretion. Since the early work by several researchers, dysregulation of glucagon secretion has also been implicated in the pathophysiology of type 2 diabetes mellitus (T2DM) [1, 2]. It is reported that fasting glucagon level is higher in T2DM compared to subjects with normal glucose tolerance (NGT) [3, 4]. Inappropriate glucagon increase in the early phase of oral glucose tolerance test, and delayed glucagon suppression has been observed in diabetes patients [5-9], compared to immediate suppression of glucagon after glucose ingestion in the subjects with normal glucose tolerance. In normal physiology, a variety of factors have been known to influence glucagon regulation, such as insulin [10-12], GLP-1 [13-19], amino acids [20-24], autonomic nervous system [25-27], free fatty acids [28], and Zn²⁺ ion [29]. However, exact mechanisms of glucagon dysregulation in T2DM patients have not yet been elucidated.

One of the major obstacles for the clinical study of glucagon dysregulation in human T2DM physiology has been the lack of precise glucagon assay to quantify its plasma levels. Glucagon has been conventionally measured by radioimmunoassay (RIA), but the sensitivity and specificity of conventional RIA kit has been accused to be unreliable [30], especially in the low concentration range [31]. A sandwich enzyme-linked immunosorbent assay (ELISA) kit, which employing two specific antibodies raised against N-terminal and C-terminal sequences of glucagon molecule, has become commercially available (released by Mercodia). It was recently confirmed that the plasma glucagon levels measured by Mercodia sandwich ELISA were well correlated with those measured by quantitative mass spectrometry (LC-MS/MS) results [32]. Because our current understanding of the pathophysiological roles for endogenous glucagon in T2DM has been based on the hundreds of...
previously published reports utilized previous immunological measurements, we set out to re-evaluate and re-define whether ‘dysregulation of glucagon secretion’ in T2DM is still valid.

Recent reports suggests that glucose tolerance deteriorates gradually from NGT to impaired glucose tolerance (IGT), ultimately leading to the development of T2DM with decreased insulin sensitivity and degradation of β-cell function [33, 34]. To clarify whether glucagon is involved in the pathophysiology of diabetes mellitus, we carried out 75 g oral glucose tolerance test (OGTT) in Japanese adult subjects and classified them as normal glucose tolerance (NGT), borderline diabetes mellitus defined as pre-diabetes mellitus (preDM) and diabetes mellitus (DM), and compared the changes in plasma glucagon concentration of NGT, preDM and DM subjects together with insulin, C-peptide immunoreactivity (CPR) and active GLP-1 levels during OGTT.

Materials and Methods

Participants

The present research was carried out with 18 volunteers, 16 patients who visited our hospital to purpose of screening for glucose metabolism abnormality, 19 patients clinically diagnosed as T2DM who are on diet therapy and exercise therapy without any anti-diabetic drugs. The inclusion criteria were as follows; age ≥20 year-old, and for patients with T2DM, glycated hemoglobin (HbA1c) <7.5% and fasting plasma glucose <140 mg/dL (7.8 mmol/L). Exclusion criteria were as follows; patients with malignancy, liver dysfunction (AST >100 IU/L and/or ALT >100 IU/L), renal impairment (estimated GFR <60 mL/min/1.73 m²) [35], pregnant or possibility of pregnancy, lactating mothers, patients suffering from endocrine disorders, patients administrated any drugs known to affect glucose metabolism, past history of gastrointestinal operation, chronic pancreatitis, insulin dependent diabetes mellitus (IDDM), diabetic patients who need any anti-diabetic drugs and are accompanied with marked diabetic complications. Written informed consent was obtained from all participants before undergoing study procedures. This study was approved by the Kitasato University Medical Ethics Organization (KMEO), Institutional Review Board for Clinical Research and Treatment (registration number C14-849) and was registered with the University Hospital Medical Information Network Center (UMIN 000016133).

Methods

Oral glucose tolerance test

All participants performed 75 g OGTT after 12 hours over night fast. Before performing OGTT, height and body weight was measured. Body mass index (BMI) was calculated from height (m) and body weight. Blood samples were collected before, 30, 60, 90 and 120 min after glucose intake for measuring plasma glucose (PG), insulin, C-peptide immunoreactivity (CPR), glucagon, and active GLP-1 (6-36-GLP-1 and 7-36-GLP-1 amide). Blood was drawn from the catheter placed in participants’ forearm or cubital vein. Blood samples for measuring glucagon and GLP-1 concentration were collected in vacutainer tubes, P800, containing EDTA-2K, protease, esterase, and dipeptidyl peptidase-4 inhibitors (Becton, Dickinson and Company, New Jersey, USA). After blood collection, the tubes were immediately immersed in ice water and centrifuged at 20°C for 20 min. Plasma was collected and stored in deep-freezer at −80°C until analysis. We used the criteria of glucose tolerance in OGTT according to World Health Organization criteria; fasting PG (FPG) <110 mg/dL (6.1 mmol/L) and PG at 120 minutes (2 h PG) <140 mg/dL (7.8 mmol/L) defined as NGT, 110 mg/dL ≤ FPG < 126 mg/dL (7.0 mmol/L) and/or 140 mg/dL (7.8 mmol/L) ≤ 2 h PG < 200 mg/dL (11.1 mmol/L) as preDM, fasting PG ≥126 mg/dL (7.0 mmol/L) and/or 2 h PG ≥200 mg/dL (11.1 mmol/L) as DM.

Biochemical analyses and calculations

PG was measured with glucose oxidase (GOD) method (Glucose analyzer GA08II, A&T Corporation, Kanagawa, Japan). Insulin was measured with electromagnetic luminescence immunoassay (ECLIA) method (Cobas 8000, Roche Diagnostics, Basel, Switzerland) and RIA assay. CPR was measured with chemiluminescent enzyme immunoassay (CLEIA) method (Presto II, FUJIREBIO INC, Tokyo, Japan). Plasma glucagon was measured by glucagon enzyme-linked immunoabsorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). The inter- and intra-CV of this glucagon ELISA kit was 7.3 to 9.4% and 3.3 to 5.1% respectively. Active GLP-1 (6-36-GLP-1 and 7-36-GLP-1 amide) was measured by using GLP-1ELISA kit (EGLP-35K, Merk Millipore, Billerica, Massachusetts, USA). In order to compare with the glucagon value measured by the ELISA kit, another glucagon measurement by the conventional RIA method (GL-32k, Merk Millipore Billerica, Massachusetts, USA) was carried out, and the glucagon value measured by this RIA method was used only for comparison with glucagon value measured by Mercodia kit.

To compare the relative glucagon secretion for PG, and insulin, CPR, GLP-1 which affecting plasma glucagon level, the ratio of each factor to glucagon was compared between each NGT, preDM, and DM group.

We used HOMA-IR [36] and Matsuda index [37] as index of insulin sensitivity. We also used insulinogenic index [38], HOMA-β [36] and oral disposition index.
Glucose disposal and the responses of insulin, CPR, glucagon, GLP-1 to oral glucose load

At all the measurement points, PG was lowest in NGT whereas highest in DM group, and there were significant differences among the three groups. The peak PG after oral glucose load became higher and delayed in the order of glucose tolerance (Fig. 1A). Insulin response exhibited a peak at 30 min in NGT group, while sustained insulin increase was observed in preDM and DM group (Fig. 1B). CPR levels exhibited similar response to insulin in the three groups (Fig. 1C).

The fasting glucagon measured by ELISA was highest in DM group followed by preDM group and by NGT group (Fig. 1D). Fasting glucagon level in DM group was 2 folds higher than NGT group and fasting glucagon level in preDM group was 1.7 folds higher than NGT group. There was significant difference in glucagon between DM group and NGT group at 0, 30, 60, and 90 min, and also between preDM and NGT group at 30, 60, and 90 min. In NGT and preDM group, glucagon secretion was highest at fasting and continuously decreased after oral glucose load. Glucagon levels in DM group was not suppressed at 30 min and then started to decrease. When conventional RIA assay was used for glucagon measurement, the glucagon excursion of NGT group and preDM group overlapped and that of DM group was slightly higher without statistical significance (Fig. 1E).

There was no significant difference among the three groups as for the GLP-1 levels at any time point. The excursion of the GLP-1 level in NGT and preDM group was parallel and peaked at 30 min, with consistently higher levels in preDM group than NGT group (Fig. 1F). GLP-1 level in DM group was lowest at fasting and increased to reach the peak at 30 min. The increment of GLP-1 from fasting to 30 min was largest in DM group, but there was no statistically significant difference between these three groups.

We analyzed the absolute glucagon change from the fasting level and relative glucagon compared to the fasting level during oral glucose load in the three groups. Fig. 2A shows the absolute glucagon change from the fasting level in each group. There was significant difference between NGT and DM group at 30 min after oral glucose intake, and between NGT, preDM group and DM group at 90 and 120 min after oral glucose intake. Fig. 2B show the relative glucagon level compared to the fasting level in each group. Similar to absolute glucagon change, there was significant difference between NGT...
and DM group at 30 min, and between NGT, preDM group and DM group at 120 min.

**Relative glucagon levels to PG, insulin, CPR, or GLP-1 levels**

Glucagon/PG ratio in DM group was significantly higher than NGT group at 30 min after glucose load (Fig. 3A). Glucagon/insulin ratio in DM group was significantly higher than NGT and preDM group at 30 min, and was significantly higher than preDM group at 90 min. Glucagon/insulin ratio in preDM group was significantly lower than NGT group at 120 min (Fig. 3B). Glucagon/CPR ratio was significantly different among the three groups at 30 min; highest in DM group followed by preDM group and then by NGT group (Fig. 3C). Glucagon/CPR ratio in DM group was significantly higher than both preDM group and NGT group at 60 min and at 90 min. Glucagon/GLP-1 ratio of DM group was significantly higher than NGT group before and at 120 min after glucose load (Fig. 3D).

**Table 1 Clinical characteristics and study population of participants**

<table>
<thead>
<tr>
<th></th>
<th>NGT (N = 25)</th>
<th>preDM (N = 15)</th>
<th>DM (N = 13)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>36.7 ± 14.6</td>
<td>54.1 ± 11.5</td>
<td>60.8 ± 4.6</td>
<td>&lt;0.01</td>
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<tr>
<td>Female</td>
<td>13</td>
<td>7</td>
<td>4</td>
<td>0.45</td>
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<tr>
<td>Height (cm)</td>
<td>164.8 ± 9.5</td>
<td>162.1 ± 9.4</td>
<td>161.9 ± 9.8</td>
<td>0.69</td>
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<tr>
<td>Body weight (kg)</td>
<td>62.7 ± 24.6</td>
<td>72.0 ± 26.8</td>
<td>67.6 ± 13.1</td>
<td>0.47</td>
</tr>
<tr>
<td>Body mass index</td>
<td>22.6 ± 6.7</td>
<td>26.9 ± 7.6</td>
<td>25.1 ± 3.8</td>
<td>0.12</td>
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<tr>
<td>Glycated hemoglobin (%)</td>
<td>5.5 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>6.4 ± 0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>91.1 ± 10.6</td>
<td>102.2 ± 10.2</td>
<td>117.9 ± 13.6</td>
<td>&lt;0.01</td>
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<tr>
<td>2 h PG (mg/dL)</td>
<td>111.3 ± 15.8</td>
<td>173.5 ± 22.9</td>
<td>252.6 ± 39.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUCPG (×10^3)</td>
<td>15.2 ± 1.8</td>
<td>21.0 ± 1.9</td>
<td>27.6 ± 4.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>6.3 ± 4.4</td>
<td>8.7 ± 4.4</td>
<td>8.4 ± 5.7</td>
<td>0.23</td>
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<tr>
<td>AUCins (×10^3)</td>
<td>5.0 ± 2.8</td>
<td>7.3 ± 4.0</td>
<td>4.6 ± 3.2</td>
<td>0.05</td>
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<tr>
<td>Fasting CPR (ng/mL)</td>
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<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>0.03</td>
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<td>HOMA-IR</td>
<td>1.4 ± 1.2</td>
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<tr>
<td>HOMA-β</td>
<td>91.8 ± 64.6</td>
<td>87.4 ± 57.0</td>
<td>56.7 ± 37.6</td>
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<td>Insulinogenic index</td>
<td>1.0 ± 1.2</td>
<td>0.6 ± 0.4</td>
<td>0.3 ± 0.4</td>
<td>0.06</td>
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<td>Matsuda index</td>
<td>7.5 ± 3.4</td>
<td>3.8 ± 1.3</td>
<td>4.9 ± 3.3</td>
<td>&lt;0.01</td>
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<td>Oral disposition index</td>
<td>2.1 ± 0.9</td>
<td>1.2 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>&lt;0.01</td>
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<td>AST (U/L)</td>
<td>20 ± 6</td>
<td>23 ± 9</td>
<td>21 ± 6</td>
<td>0.35</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>20 ± 8</td>
<td>23 ± 9</td>
<td>19 ± 7</td>
<td>0.32</td>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>74 ± 34</td>
<td>200 ± 320</td>
<td>153 ± 95</td>
<td>0.09</td>
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<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>67 ± 16</td>
<td>65 ± 13</td>
<td>58 ± 21</td>
<td>0.24</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dL)</td>
<td>112 ± 32</td>
<td>118 ± 27</td>
<td>122 ± 26</td>
<td>0.53</td>
</tr>
</tbody>
</table>

† p < 0.05 vs. NGT  ‡ p < 0.05 vs. preDM

**AUC of PG, insulin, CPR, glucagon and GLP-1 during OGTT**

As expected, AUCPG significantly increased in the order of glucose tolerance from NGT, preDM, and to DM (Fig. 4A). AUCins and AUCGLP-1 were not significantly different among the three groups (Fig. 4B and 4E), although significant differences in AUCCPR between NGT and preDM, or between preDM and DM were observed (Fig. 4C). Importantly, AUCgk of DM was significantly larger than NGT and preDM group (Fig. 4D). Also, AUCgk of preDM tended to be larger than NGT group (p < 0.05).

**Correlation between fasting glucagon and insulin sensitivity and correlation between fasting glucagon and pancreatic β-cell function**

There was significant positive correlation between fasting glucagon (containing all participants’ value) and indexes of insulin resistance; fasting glucagon positively correlated to HOMA-IR (Fig. 5A) and negatively correlated to Matsuda index (Fig. 5B). On the other hand,
there was no significant correlation between fasting glucagon and indexes of pancreatic β-cell function including insulinogenic index and HOMA-β (Fig. 5C and 5D) except for the significant negative correlation between fasting glucagon and oral DI (Fig. 5E). When examining the relationship between these five parameters and fasting glucagon in each group of NGT, preDM, DM, no significant correlation was observed. There was significant and strong positive correlation between fasting glucagon and AUC_{glc} (Supplemental Fig. 1). This strong correlation was also observed when examined in each group.

Relationship between plasma glucagon level during OGTT and other clinical parameters

There was significant correlation between BMI and glucagon at 30 min, significant correlation between glucagon and HbA1c at fasting, 30 min, and 90 min. There was significant correlation between fasting TG and glucagon at all time points during the oral glucose load. We did not observe significant correlation between fasting AST or ALT and glucagon at any time point (Supplemental Fig. 2).

Fasting glucagon and glucagon excursion during OGTT in each individual subject

Fig. 5 and Supplemental Fig. 1 show that fasting glucagon varies more as glucose intolerance develops. To understand the individual variability of glucagon levels, glucagon excursion during OGTT in each individual subject in the three groups was plotted (Fig. 6).

In NGT group, fasting glucagon clustered in a narrow range (ranges between 5–50 pg/mL, mostly between 5–40 pg/mL). After glucose load, glucagon started to decrease in most of the individuals in this group (Fig. 6A). As glucose intolerance develops, the distribution of fasting glucagon shifted to upper value and the range widened. In the preDM group (Fig. 6B), fasting glucagon ranged between 10–90 pg/mL, mostly between 10–50 pg/mL, and in the DM group (Fig. 6C) it ranges between 10–90 pg/mL, and evenly distributed in this range.

The fraction of individuals whose glucagon level increased after oral glucose load increased with worsening of glucose tolerance (data not shown). To elucidate the factors related to glucagon increment at 30 min after glucose load, all participants were divided into the group whose glucagon increased at 30 min and that whose glucagon decreased at 30 min. We examined whether the change in PG (ΔPG), insulin (Δinsulin), CPR (ΔCPR) and GLP-1 (ΔGLP-1) at first 30 min after glucose intake differed between these two groups. We also examined whether the index of insulin sensitivity and index of pancreatic β-cell function differed between the two groups. In the glucagon increased group, Δinsulin and ΔCPR were significantly lower than the glucagon decreased group (Supplemental Fig. 3B and 3C), whereas no significant difference was found in ΔPG and ΔGLP-1 between two groups (Supplemental Fig. 3A and 3D). Although insulinogenic index, HOMA-β, Oral DI were lower in the glucagon increased group than the glucagon decreased group, statistical significance was only observed in Oral
In this study we found that fasting glucagon level increased with worsening of glucose tolerance, from NGT, borderline diabetes mellitus and to mild T2DM using an accurate glucagon ELISA assay. Fasting glucagon level in DM group was significantly elevated (2 folds higher) compared to NGT group and that in preDM group was 1.7 folds higher (although not significantly) than NGT group. There was no significant difference in fasting insulin levels and fasting CPR among the three groups. Early glucagon suppression at 30 min was not observed in mild T2DM patients and less suppressed in borderline diabetes subjects compared to NGT subjects. On the other hand, fasting GLP-1 levels and excursion after oral glucose load was not significantly different among the three groups.

The fasting glucagon doubled in mild T2DM compared to NGT subjects in our study. In the report with Caucasian participants, fasting glucagon in patients with T2DM was 1.3 times higher than that of NGT subjects [7]. These data suggest the importance of elevated glucagon and resulting increased hepatic glucose output in the pathophysiology of mild T2DM in Japanese subjects.

Although RIA method is an excellent measurement method with high detection sensitivity and specificity for trace compound, it has been pointed out that there are serious problems in commercially-available RIA kit for glucagon in its sensitivity and specificity [31]. The glucagon ELISA kit released from Mercodia shows high sensitivity and specificity to glucagon compared to conventional commercially-available RIA kits [30]. In our study, while glucagon assay using the ELISA kit revealed elevated fasting glucagon in DM group and inappropriate glucagon suppression after oral glucose load in preDM and DM group, glucagon assay using a conventional RIA kit was not sensitive or specific enough to reveal these findings (Fig. 1D and 1E). These results support the high reproducibility and reliability of this ELISA kit.

**Elevated fasting glucagon in subjects with impaired glucose tolerance**

In this study, as in the previous report [5-9], it was found that the average of fasting glucagon and FPG became higher with worsening of glucose tolerance, but there was no significant difference in fasting insulin and CPR level among NGT, preDM and DM group. Significant correlation was found between fasting glucagon level and the indicators of insulin sensitivity such as HOMA-IR and Matsuda index. On the other hand, there was no correlation between fasting glucagon level and indicators of β-cell function such as insulinogenic index and HOMA-β. There are two possible explanations to understand the relationship between elevated fasting glucagon and attenuated insulin sensitivity without the influence of β-cell function. One is that attenuated insulin sensitivity at the islet level may be responsible for the elevation of fasting glucagon [40, 41]. Another explanation is that elevated fasting glucagon is one of the pathophysiology of borderline diabetes and mild T2DM independently from insulin dysregulation. In the former explanation we hypothesize the insulin-induced suppression of glucagon secretion as paracrine effect at the islet level. Although the inhibition of glucagon secretion by insulin at the islet level has been investigated vigorously using animal models [41], there are several observations that insulin-induced glucagon suppression at the islet level is not always operative. For example, insulin and glucagon are both increased after mixed meal ingestion in normal subjects [42-44] suggesting that insulin and
glucagon are regulated in more complex manner than the simple insulin-induced inhibition of glucagon. The observation that glucose induced inhibition of glucagon in diabetes subjects is observed only when glucose was introduced orally but not parenterally that has been explained by incretins [5] also suggests that insulin-induced suppression of glucagon at the islet level is not necessarily operative. If we employ the latter explanation, we can explain that the elevated fasting glucagon inducing hepatic glucose output contributes to insulin

Fig. 3  Relative glucagon levels to PG (A), insulin (B), CPR (C), or GLP-1 (D) levels during OGTT in NGT, preDM and DM group. * p < 0.05.

Fig. 4  Comparison of area under the curve (AUC) of PG (A), insulin (B), CPR (C), glucagon (D) and GLP-1 in NGT (open bar), preDM (grey bar) and DM (closed bar). Data are mean ± SEM. * p < 0.05 vs. NGT, ** p < 0.05 vs. preDM.
resistance at the liver level and do not have to hypothesize the insulin resistance at the islet level; fasting insulin is elevated partly to compensate for the glucagon-induced hepatic glucose output. It has been suggested that both the abnormality in insulin action and secretion and glucagon secretion are involved in the pathogenesis of T2DM [1]. We suggest that elevation in fasting glucagon occurs independently in borderline diabetes and mild T2DM and may partly contribute to insulin resistance in borderline diabetes and mild T2DM. In our study, preDM and DM group not only showed elevated fasting glucagon level compared to NGT group, also demonstrated greater glucagon suppression after glucose load (Fig. 1D), and delayed and larger insulin secretion in preDM and DM group compared to NGT group (Fig. 1B) might be involved in this greater glucagon suppression by oral glucose load. It was considered these factors resulted relative glucagon suppression in preDM and DM group after glucose intake compared to NGT group. There was significant correlation between HbA1c and glucagon at fasting and during the oral glucose load in the participants is in consistent with the discussion above. We observed significant cor-

Fig. 5 Correlation between fasting glucagon and HOMA-IR (A), Matsuda index (B), HOMA-β (C), insulogenic index (D) and Oral DI (E). Fasting glucagon is significantly correlated to HOMA-IR, Matsuda index and Oral DI, but these significant correlations disappeared when examining in each NGT (open circle), preDM (open square) and DM group (closed diamond).

Fig. 6 Glucagon fluctuation during OGTT in individual subjects with NGT (A), preDM (B) and DM (C) plotted separately. As glucose metabolism abnormality developed, the range of glucagon excursion expanded and the proportion of subjects with high fasting glucagon level increased.
relation between fasting TG and glucagon at fasting and during oral glucose load. This finding may suggest that TG metabolism or liver steatosis that induce elevation in TG is related to or has causative relation to the elevated glucagon level in patients with glucose intolerance.

**Defective early suppression of glucagon in T2DM**

Another finding in this study was that early glucagon suppression was defective in the DM group. Glucagon/insulin or glucagon/CPR ratio increased significantly with worsening of glucose intolerance (Fig. 3). As mentioned above, it is suggested both abnormalities of insulin and glucagon secretion were independently involved in the pathophysiology of T2DM. In DM group, fasting glucagon level was inappropriately elevated and immediate and adequate glucagon suppression was not observed after oral glucose intake as NGT and preDM group demonstrated. Moreover, insulin secretion in early phase of OGTT was reduced and response to increase insulin secretion was delayed in DM group due to decreased pancreatic beta cell function compared to NGT and preDM group. As a result of combining these factors, it is considered that relative glucagon ratio to PG, insulin and CPR was significantly elevated in DM group at 30 min after oral glucose load compared to the other two groups. Several studies reported the inappropriate glucagon increment during the early phase after oral glucose ingestion in diabetic subjects, and this glucagon increment was observed when glucose was applied orally but not by intravenously [5]. It has been considered that gastrointestinal-derived hormones, GLP-1, GIP and GLP-2, are involved in this glucagon increment [18]. It has been experimentally shown that GLP-1 suppresses glucagon secretion [16], and a previous report indicated that total GLP-1 secretion decreased with deterioration of glucose metabolism [45]. However there are also negative opinions that there is no difference in GLP-1 and GIP secretion between subjects with type 2 diabetes and healthy individuals, and that both hormones are not involved in inappropriate glucagon increment in diabetic subjects [46], consistent with our results. Experimental data using mice have shown that GLP-1 in pancreatic islets rather than systemic circulating GLP-1 is involved in the compensatory mechanism of pancreatic β-cells to insulin resistance [47]. Further studies are necessary to elucidate whether defective glucagon suppression explained by the action of GLP-1 at the islet level.

**Variability of glucagon response among subjects**

We additionally analyzed the fluctuation of glucagon in individual cases in all the groups. As shown in Fig. 6, glucagon value in all the subjects in NGT group was almost less than 40 pg/mL at all measurement points and they fluctuated within a narrow range. On the other hand, the variation of glucagon became larger as glucose intolerance developed. Furthermore, focusing on the glucagon elevation after 30 minutes of glucose load as shown in Fig. 1 which is “characteristic” of diabetes cases, it was found that this glucagon elevation was not necessarily observed in all the diabetic subjects. We also found that there are some cases in which glucagon increases after oral glucose intake even in subjects with normal glucose tolerance (Fig. 6). There were a number of subjects in preDM and DM group whose fasting glucagon value was as low as the subjects with normal glucose tolerance and whose glucagon value fluctuated within a narrow range similarly to subjects of NGT group during OGTT. This finding suggests that the contribution of glucagon dysregulation is different in each individual with altered glucose tolerance.

In conclusion, subjects with mild T2DM showed elevated fasting glucagon and paradoxical glucagon increase after oral glucose load compared to subjects with normal glucose tolerance. The elevated fasting glucagon was associated with decreased insulin sensitivity, and inappropriate glucagon increase after oral glucose load was associated with decreased insulin secretion, but not with GLP-1 secretion.

**Disclosure**

None of the authors have any potential conflicts of interest associated with this research.
Supplemental Fig. 1  Relationship between fasting glucagon and area under the curve of glucagon (AUC_{glc}) in all participants, and in subjects with NGT (open circle), preDM (open square) and DM (closed diamond), separately. Significant and positive correlation was observed between fasting glucagon and AUC_{glc}, and this significant positive correlation is also observed when examining in each NGT, preDM and DM group.

Supplemental Fig. 2  Relationship between BMI (upper panel), HbA1c (middle panel), and TG (bottom panel) and plasma glucagon levels during OGTT in all participants, and in subjects with NGT (open circle), preDM (gray square) and DM (closed circle), separately. TG showed significant positive correlation with plasma glucagon level at all measurement points, and this significant positive correlation was still observed when examining separately in preDM and DM group, but not in NGT group. BMI showed significant but weak positive correlation with plasma glucagon level at 30 min, and HbA1c showed at 30 and 60 min. In these parameters, no significant correlation was observed when examining separately in each NGT, preDM, and DM group.
Supplemental Fig. 3  Comparison of increment of glucose (A), insulin (B), CPR (C) and GLP-1(D) during the first 30 minutes after glucose load between subjects whose glucagon increased or decreased at 30 minutes after oral glucose load. Significant difference was observed in insulin increment and CPR increment between the two groups. ※ p < 0.05.

Supplemental Fig. 4  Comparison of index of insulin sensitivity (upper panel) and pancreatic β-cell function (lower panel) between subjects whose glucagon increased or decreased at 30 minutes after oral glucose load. There was little difference in HOMA-IR (A) and Matsuda index (B) between the two groups. Whereas insulinogenic index (C), HOMA-β (D) and Oral DI (E) tended to be lower in subjects whose glucagon decreased, with significant difference only in Oral DI. ※ p < 0.05.
References


