Estimated proinsulin processing activity of prohormone convertase (PC) 1/3 rather than PC2 is decreased in pancreatic β-cells of type 2 diabetic patients

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Abstract. Type 2 diabetic (T2D) patients exhibit fasting relative hyperproinsulinemia owing to pancreatic β-cell dysfunction. To clarify the mechanism underlying this hyperproinsulinemic state, we evaluated the activities of the endopeptidases prohormone convertase (PC) 1/3 and PC2 in T2D patients. Fasting blood levels of intact proinsulin (IPI), total proinsulin (t-PI) and C-peptide were measured simultaneously, and intravenous glucagon loading was performed to investigate the dynamics of circulating proinsulin-related molecules released from pancreatic β-cells in 12 healthy volunteers and 18 T2D patients. Taking advantage of the 95% cross-reactivity between proinsulin and des-31,32-proinsulin (des-31,32-PI) with the human proinsulin radioimmunoassay kit used in this study, we estimated PC1/3 and PC2 activities using the following formulas: des-31,32-PI = (t-PI–IPI)/0.95; PC1/3 activity = des-31,32-PI/IPI; and PC2 activity = C-peptide/des-31,32-PI. C-peptide responses to glucagon were slightly lower among T2D patients. IPI and the IPI/C-peptide ratio were significantly higher in T2D patients (p<0.05 and p<0.01, respectively). There was no difference in des-31,32-PI levels or PC2 activity between the two groups. However, PC1/3 activity was significantly lower in T2D patients than in the control group (p<0.01). We propose that decreased activity of PC1/3 rather than PC2 in pancreatic β-cells is involved in the impaired proinsulin processing, resulting in elevated IPI levels in T2D patients.

Key words: Insulin secretion, Insulin biosynthesis, Proinsulin

PATIENTS with type 2 diabetes show impaired acute-phase insulin secretion in response to glucose as well as fasting relative hyperproinsulinemia owing to pancreatic β-cell dysfunction [1]. This condition is known to develop in subjects with mild fasting hyperglycemia of 6.1–7.8 mM [2], but the factors involved and the underlying pathophysiological mechanisms are not well understood.

Insulin and C-peptide are both derived from a common precursor molecule, proinsulin. There are two dibasic cleavage sites in human proinsulin: Arg31-Arg32 and Lys64-Arg65. Proteolytic processing of proinsulin yields mature insulin by cleaving C-peptide, which separates the A and B chains of proinsulin. Two endopeptidases, prohormone convertase (PC) 1/3 and PC2 are involved in the excision of C-peptide; the former specifically cleaves proinsulin at Arg31-Arg32 and the latter preferentially cleaves proinsulin at Lys64-Arg65.

The predominant processing pathway in β-cells first converts proinsulin to the intermediate des-31,32-proinsulin (des-31,32-PI) following cleavage by PC1/3, and subsequently to insulin and C-peptide following cleavage by PC2 [3]. Des-31,32-PI is the preferred substrate of PC2, and mice lacking active PC2 have elevated levels of proinsulin and des-31,32-PI [4]. Despite this, approximately two-thirds of proinsulin was converted to mature insulin in these mice. By contrast, mice lacking PC1/3 showed elevated levels of another intermediate, des-64,65-proinsulin (des-64,65-PI), which is generated by PC2 cleavage, and caused more severe
blockage of proinsulin processing than did the absence of PC2 [5]. Thus, it appears that while the coordinated activity of both endopeptidases is essential for normal processing of proinsulin, PC1/3 contributes quantitatively more to this process than does PC2. Consistent with this, severe hyperproinsulinemia and high levels of des-64,65-PI were observed in a woman with mutations in PC1 [6].

Several different proinsulin-specific radioimmunoassays (RIAs) have been used to measure circulating proinsulin levels. Among them, the human proinsulin RIA kit (LINCO Research, Inc., St. Charles, MO, USA) shows strong cross-reactivity with des-31,32-PI as well as intact proinsulin. Recently, a new chemiluminescence assay showing virtually no cross-reactivity with des-31,32-PI and good specificity for intact proinsulin, as described in the kit manual, was developed [7]. In this study, we measured the fasting levels of C-peptide, intact proinsulin, and des-31,32-PI simultaneously in type 2 diabetic patients and healthy volunteers using both of these methods. Intravenous glucagon loading was also performed to investigate the dynamics of circulating proinsulin-related molecules released from insulin secretory granules. We then estimated the relative activities of PC1/3 and PC2 based on the levels of proinsulin-related molecules to determine which cleavage site in proinsulin is predominantly involved in impaired proinsulin processing in type 2 diabetic patients.

**Materials and Methods**

**Subjects**

The study group comprised 12 healthy subjects aged 28–52 years and 18 type 2 diabetic subjects aged 27–73 years. The mean duration of diabetes in these subjects was 9.1±2.3 years. None of the subjects had renal dysfunction. Each subject gave written, informed consent for participation in the study, and the study was carried out in accordance with the Declaration of Helsinki. Ethical guidelines had not been announced by the Ministry of Health, Labour and Welfare at the time of this study. Therefore, ethics committee approval was not obtained.

**Clinical evaluation**

All subjects were examined the morning after a 10-h overnight fast. Thirty min after placement of an indwelling cannula in a forearm vein, basal blood samples were drawn for measurement of various proinsulin-related molecules, including C-peptide, insulin, total proinsulin (t-PI, which includes all of its major processed intermediates), and intact proinsulin (IPI). Blood samples were taken immediately before and 3 and 6 min after intravenous injection of 1 mg of glucagon (Novo Nordisk, Copenhagen, Denmark). Samples for glucose determination were collected in tubes containing fluoride and heparin; those for C-peptide, insulin, t-PI, IPI determination were collected in tubes containing EDTA with 1,000 kallikrein inhibitor units aprotinin/mL whole blood. Glucose samples were sent immediately to the laboratory for measurements. Other samples were frozen and kept at −70°C until assayed.

**Assays**

Plasma glucose level was determined by the glucose oxidase method (Beckman Instruments, Fullerton, CA, USA). Serum C-peptide level was determined using a C-peptide immunoradiometric assay kit (Eiken Chemical, Co. Ltd, Tokyo, Japan). Serum insulin level was measured using the monoclonal insulin ELISA technique with an AxSYM insulin kit (Dinabot, Tokyo, Japan) [8]. IPI was assessed using an intact proinsulin chemiluminescence assay kit (Molecular Light Technology Research Ltd., Cardiff, UK). t-PI was assayed using a human proinsulin RIA kit (LINCO Research, Inc.) [7]. All samples from type 2 diabetic patients were measured in the same run as samples from at least one of the healthy controls.

**Calculations**

The minimum detectable sensitivity of the C-peptide assay was 3.3 pmol/L, and the inter-assay and intra-assay coefficients of variation (CVs) were 5.8% and 5.1%, respectively. The minimum detectable sensitivity of the insulin assay was 1.0 pmol/L, and the inter-assay and intra-assay CVs were 5.2% and 3.6%, respectively. The minimum detectable sensitivity of the IPI assay system was 0.6 pmol/L, and the inter-assay and intra-assay CVs were 8.6% and 5.2%, respectively. The minimum detectable sensitivity of the t-PI assay was 2.0 pmol/L, and the inter-assay and intra-assay CVs were 8.6% and 5.2%, respectively. A 95% cross-reactivity of the t-PI assay with des-31,32-PI has been reported [7]. None of these proinsulin measurement systems showed any cross-reactivity with C-peptide or insulin. Assay characteristics are reported from the manufacturer’s instructions for each assay.
The levels of circulating des-31,32-PI were estimated using the following formula:  
\[
\text{des-31,32-PI} = \frac{\text{t-PI} - \text{IPI}}{0.95}.
\]

Because proinsulin is first predominantly cleaved to the intermediate des-31,32-PI by PC1/3, and des-31,32-PI is then cleaved to insulin and C-peptide by PC2, we estimated PC1/3 and PC2 activities using the following formulas:  
\[
\text{PC1/3 activity} = \frac{\text{des-31,32-PI}}{\text{IPI}},
\]
\[
\text{PC2 activity} = \frac{\text{C-peptide}}{\text{des-31,32-PI}}.
\]

For all variables measured during the glucagon loading test, the area under the curve for 0–6 min (AUC$_{0-6\text{min}}$) was calculated using the trapezoidal method.

**Statistical analyses**

Measurements are expressed as means ± standard error (S.E.). StatView version 5.0 (Abacus Concepts Inc., Berkeley, CA, USA) was used for statistical analysis, and the significance of differences in mean values between groups was analyzed using Fisher’s PLSD multiple comparison test. The level of significance was set at 5% and a p-value of less than 0.05 was considered to represent statistical significance.

**Results**

**Alterations in the levels of circulating proinsulin-related molecules in type 2 diabetic patients in the fasted state**

Table 1 shows the characteristics of type 2 diabetic patients and healthy controls in the fasted state. Body mass index was slightly, although not significantly, higher in type 2 diabetic patients than in healthy controls. Glucose levels, HbA1c, HOMA-R, IPI levels, and the IPI/C-peptide ratio were significantly higher in patients with type 2 diabetes than in healthy controls (all p<0.01). Insulin, C-peptide, and t-PI levels, and the IPI/insulin ratio were not significantly different between the two groups.

**C-peptide responses to intravenous glucagon loading in type 2 diabetic patients and healthy controls**

To ascertain the endogenous insulin secretory capacity in the 18 type 2 diabetic patients and 12 healthy controls included in our study, we performed intravenous glucagon loading in the fasted state and examined C-peptide responses. As shown in Fig. 1A, although baseline C-peptide levels were similar in both groups of subjects, the levels at 3 and 6 min and the AUC$_{0-6\text{min}}$ were slightly lower, albeit not significantly, in type 2 diabetic patients than in healthy controls.

**Variations in the levels of proinsulin-related molecules in type 2 diabetic patients after intravenous glucagon loading**

Variations in the levels of proinsulin-related molecules in both groups are shown in Fig. 2. t-PI levels in type 2 diabetic patients were similar to those in healthy controls (Fig. 2A, B). The IPI levels were higher in type 2 diabetic patients at 0, 3, and 6 min during the intravenous glucagon test. Although the differences were not significant at each time point (Fig. 2C), the AUC$_{0-6\text{min}}$ for IPI was significantly higher in type 2 diabetic patients than in healthy controls (p<0.05).

We then evaluated the IPI/C-peptide ratios in both groups. The calculated IPI/C-peptide ratios were significantly higher in type 2 diabetic subjects than in control subjects at all time-points examined (Fig. 2E), as was the AUC$_{0-6\text{min}}$ for the IPI/C-peptide ratio (p<0.01; Fig. 2F).

**Table 1** Characteristics of type 2 diabetic patients and healthy controls in the fasted state

<table>
<thead>
<tr>
<th>n</th>
<th>Healthy controls</th>
<th>Type 2 diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>22.6±0.9</td>
<td>25.0±0.8</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>92.6±2.1</td>
<td>131.8±11.1**</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.83±0.10</td>
<td>9.89±0.48**</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>31.4±2.7</td>
<td>46.1±5.4</td>
</tr>
<tr>
<td>C-peptide (pmol/L)</td>
<td>457.1±45.7</td>
<td>509.9±47.0</td>
</tr>
<tr>
<td>HOMA-R</td>
<td>1.2±0.1</td>
<td>2.4±0.3**</td>
</tr>
<tr>
<td>IPI (pmol/L)</td>
<td>11.8±1.4</td>
<td>14.6±2.0</td>
</tr>
<tr>
<td>IPI/insulin ratio</td>
<td>0.127±0.020</td>
<td>0.185±0.026</td>
</tr>
<tr>
<td>IPI/C-peptide ratio</td>
<td>0.009±0.001</td>
<td>0.014±0.001**</td>
</tr>
<tr>
<td>Antidiabetic drugs, n†</td>
<td>Sulfonylurea</td>
<td>n/a</td>
</tr>
<tr>
<td>Metformin</td>
<td>n/a</td>
<td>3</td>
</tr>
<tr>
<td>α-glucosidase inhibitor</td>
<td>n/a</td>
<td>2</td>
</tr>
<tr>
<td>Insulin</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>n/a</td>
<td>2</td>
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<tr>
<td>Unknown</td>
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<td>4</td>
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</table>

Data are expressed as means ± SE. *p<0.05, **p<0.01 vs. healthy controls. †Some patients were using multiple drugs. HbA1c, hemoglobin A1c; HOMA-R, homeostatic model assessment of insulin resistance; t-PI, total proinsulin; IPI, intact proinsulin; n/a, not applicable.
The increased proinsulin/insulin ratio in type 2 diabetic patients has been attributed to a chronic increase in demand on the β-cell secretory pathway due to hyperglycemia under diabetic conditions, with newly synthesized proinsulin not being retained within insulin secretory granules long enough for complete processing to insulin [9]. Impairment of the β-cell proinsulin conversion mechanism has been suggested as the most likely cause of this hyperproinsulinemia. In particular, a coordinate increase in PC1/3 biosynthesis in response to increased demand for insulin in the hyperglycemic state, with no corresponding increase in PC2 biosynthesis, is thought to be a key mechanism underlying the hyperproinsulinemia in type 2 diabetic patients [3]. However, our results suggest that disturbed proinsulin processing itself is an important pathophysiological factor in the development of type 2 diabetes.

Intravenous glucagon loading has been used for the clinical evaluation of the endogenous maximum insulin secretory capacity owing to the increase in intracellular cAMP levels that in turn activate protein kinase A and Epac2 [10], and accelerates the exocytosis of proinsulin-related molecules from the readily releasable pool of insulin secretory granules in pancreatic β-cells. Estimated PC1/3 activity was significantly lower in type 2 diabetic patients than in healthy controls at 0
Fig. 2 Responsiveness of proinsulin-related molecules to intravenous glucagon loading in type 2 diabetic patients and healthy controls.

A, C, E: Time-course for responsiveness of total proinsulin (t-PI) (A), intact proinsulin (IPI) (C), and IPI/C-peptide ratio (E) to glucagon in type 2 diabetic patients and healthy controls. B, D, F: AUC_{0–6min} for t-PI (B), IPI (D), and IPI/C-peptide ratio (F) after glucagon stimulation in type 2 diabetic patients and healthy controls. Data are expressed as means ± S.E., *p<0.05, **p<0.01 vs. healthy controls. AUC_{0–6min}: area under the curve for 0–6 min. Healthy control group (○), type 2 diabetic patients (●).
Fig. 3  Levels of calculated des-31,32-proinsulin and PC2 and PC1/3 activities before (basal state) and after intravenous glucagon loading in type 2 diabetic patients and healthy controls. A, C, E: Time-course of calculated des-31,32-proinsulin levels (A), estimated PC2 activity (C), and estimated PC1/3 activity (E) assessed at the indicated times before and after glucagon loading. B, D, F: AUC$_{0-6 min}$ for des-31,32-proinsulin levels (B), estimated PC2 activity (D), and estimated PC1/3 activity (F) after glucagon stimulation in type 2 diabetic patients and healthy controls. Values are represented as means ± S.E., *p<0.05, **p<0.01 vs. healthy controls. AUC$_{0-6 min}$: area under the curve for 0–6 min. Healthy control group (○), type 2 diabetic patients (●).
and 6 min, and also in terms of the AUC_{0–6min} (Fig. 3E, F). These results indicate that the level of PC1/3 in the readily releasable pool of insulin secretory granules in β-cells is decreased in these patients. The C-peptide responses to glucagon loading were slightly reduced in type 2 diabetic patients, accompanied by a marked increase in IPI levels and the IPI/C-peptide ratio. It was reported that cAMP signaling might regulate the exocytotic dynamics of insulin granules located some distance away from the plasma membrane [11]. Thus, poor C-peptide responses to glucagon could reflect a decrease in the number of insulin granules caused by reduced β-cell mass in diabetic patients. Type 2 diabetes occurs because the β-cell mass and function are unable to compensate for insulin resistance [12]. We propose that intravenous glucagon loading may be useful for the clinical evaluation of altered proinsulin processing in insulin secretory granules and for the evaluation of residual pancreatic β-cell mass in type 2 diabetes. There was a small, non-significant increase in PC2 activity in type 2 diabetic patients; this increase might occur to compensate for the reduction in PC1/3 activity. However, the increase appeared to be insufficient to fully normalize circulating C-peptide levels.

The present findings should be interpreted while considering the study’s limitations. In particular, the number of patients and control subjects recruited for this study was very small, making it difficult to control for factors such as age, duration of diabetes, types of antidiabetic drugs that the patients used, renal function and HbA1c. Nevertheless, statistically significant differences were observed for a number of parameters, especially the IPI/C-peptide ratio and estimated PC1/3 activity. We must also acknowledge the possibility that the use of different assay kits based on different methods might contribute to the differences in estimated PC1/3 and PC2 activities. In addition, we could not determine the expression levels of PC1/3 and PC2 or the number of viable β-cells in diabetic patients. Some studies have also suggested that pancreatic Ca^{2+} and pH [13], oxidative stress [14], macrophage infiltration [15], and lipotoxicity [16] can affect β-cell function. Therefore, the possible effects of these factors on PC1/3 and PC2 activities should be examined in future clinical and/or animal studies. Larger-scale studies, with adjustments for factors that might influence β-cell function, should be performed in the future to confirm these findings. Further studies should also compare the metabolic clearance rates of C-peptide, IPI, and des-31,32-PI to determine whether differences in their clearance may contribute to the differences in their circulating levels.

In conclusion, decreased PC1/3 rather than decreased PC2 activity in insulin secretory granules in pancreatic β-cells contributes, at least in part, to the increased proinsulin/insulin ratio observed in type 2 diabetes patients. Further study of impaired processing will be beneficial to improve the therapeutic efficacy of insulin secretagogues.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

References


