Pancreatic Function of Spontaneously Diabetic Torii Rats in Pre-Diabetic Stage

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Abstract: The Spontaneously Diabetic Torii (SDT) rat is a new model for non-obese type 2 diabetes. In the present study, we investigated changes in insulin secretion from the pancreas of male SDT rats aged 8, 16, and 24 weeks in order to analyze pancreatic function. An analysis of glucose-stimulated insulin secretion (GSIS) in isolated islets showed a marked reduction in insulin secretion in pre-diabetic 16-week-old SDT rats. When the islets were treated with tolbutamide or glucagon-like peptide-1 (7-36) amide (tGLP-1) in the presence of 11.2 mM glucose, however, insulin levels were restored to levels of normal rats. In vivo study, SDT rats exhibited a marked reduction in GSIS from 16 weeks of age. However, tolbutamide or JTP-76209, which is a novel dipeptidyl peptidase IV (DPP IV) inhibitor, increased insulin release after glucose loading and improved glucose tolerance. A marked reduction in GSIS was observed in pre-diabetic SDT rats and the reduction was improved by tolbutamide, tGLP-1, and the DPP IV inhibitor. Therefore, we concluded that the SDT rat is useful, as a model of non-obese insulin secretory disorder, for the analysis of the onset of type 2 diabetes and the development of antidiabetic agents.

Key words: DPP IV inhibitor, GSIS, islet, pre-diabetes, SDT rat

Introduction

Multiple etiologic factors, including genetic and environmental factors, are considered to play a role in the onset of type 2 diabetes mellitus, and animal models of diabetes are very useful for understanding the mechanism of onset. At present, Zucker Fatty (ZF) rats [23], Zucker Diabetic Fatty (ZDF) rats [26], Goto-Kakizaki (GK) rats [7], Otsuka Long-Evans Tokushima Fatty (OLETF) rats [5], etc. are widely employed as rat models of type 2 diabetes mellitus in order to analyze the onset of the disease and to develop antidiabetic agents. In contrast, experimental models treated by alloxan or streptozotocin to induce specific damage to pancreatic β cells are widely used as models of diabetic complications [21]. With these experimental models, though, a direct drug effect on the rat cannot be ruled out and a new spontaneous diabetic rat model is currently desired to analyze diabetic complications.

The Spontaneously Diabetic Torii (SDT) rat is a model of non-obese spontaneous diabetes which was developed by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan).
Japan) [11, 24]. Male SDT rats develop hyperglycemia, hypoinsulinemia, and hyperlipidemia with age, and express urinary glucose from about 20 weeks of age. SDT rats also develop ocular complications (cataract and retinopathy) and nephropathy from about 40 weeks of age [24]. Sasase et al. have reported that the lesions in SDT rats are induced by hyperglycemia because an improvement is observed following continuous subcutaneous insulin administration [14, 20]. Therefore, the SDT rat is expected to be useful as a new animal model of diabetes-associated complications such as retinopathy and nephropathy.

Type 2 diabetes mellitus is a heterogeneous syndrome of polygenic origin which involves both defective insulin secretion and peripheral insulin resistance. The concepts of glucose toxicity and lipotoxicity have recently been proposed because of the role played by chronic hyperglycemia and hyperlipidemia in the onset and exacerbation of diabetes mellitus [27, 29]. Previously, diminished peripheral insulin sensitivity was widely held to be principally responsible for the onset and exacerbation of diabetes mellitus because of the inhibitory effects of hyperglycemia and hyperlipidemia on insulin action in insulin-sensitive peripheral organs (principally the liver, muscle, and fat). More recently, though, it has been reported, based on the findings of a study using pancreatic isolated islets and a pancreatic β cell line, that insulin secretion is coordinately controlled by glucose and lipids and that, when the effects of the glucose and lipids become excessive (chronic), this coordinated effect fails, leading to impaired insulin secretion and the onset or exacerbation of diabetes mellitus [17–19].

It has been reported that SDT rats develop diabetes mellitus due to morphological and functional abnormalities in the pancreas associated with aging, but the exact etiology is unclear. Masuyama et al. performed quantitative trait locus (QTL) analysis of the SDT rat and clarified that three loci, Gisdt1 (Glucose intolerance in SDT rat 1), Gisdt2, and Gisdt3, are involved in glucose intolerance on chromosomes 1, 2, and X, respectively [10]. Since these traits of exacerbated glucose tolerance are attributed to polygenic effects, an analysis of SDT rats would be highly useful in studies of the relationship between diabetes-associated genes and diabetes mellitus.

The present study focused primarily on insulin secretion from the pancreas and examined the periodic changes in pancreatic function in male SDT rats in order to analyze pancreatic function in pre-diabetic SDT rats.

**Materials and Methods**

**Animals**

Male SDT rats aged 8, 16, and 24 weeks were used in this study. Age-matched Sprague-Dawley (SD) rats were used as the control animals. SDT and SD rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). The rats were housed in a climate-controlled room with a temperature of 23 ± 3°C and a humidity of 55 ± 15% at the Central Pharmaceutical Research Institute of Japan Tobacco, Inc. All animals were fed a basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and water was provided ad libitum. All experiments were performed in accordance with the in-house Manual of Animal Experiments.

**Biochemical parameters**

In this study, to evaluate age-related changes in biological parameters in SDT and SD rats, blood samples were collected from the tail vein in non-fasted rats, to minimize the stress associated with fasting, and the plasma was obtained. Plasma glucose levels were measured using an automatic analyzer (Hitachi 7170S; Hitachi, Tokyo, Japan). Plasma insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biochemical Science, Yokohama, Japan).

**Glucose tolerance test and arginine tolerance test**

Glucose solution (0.3 g/kg) was administered via the dorsal metatarsal vein to 4-h-fasted rats for the intravenous glucose tolerance test (IVGTT). Blood samples were collected before (0 min), and 5 and 10 min after glucose loading. To evaluate the effect of dipeptidyl peptidase IV (DPP IV) inhibitor (JTP-76209; 2-{(trans-4-[(S)-amino-(N-cyclobutyl-N-methylcarbamoyl)methyl)cyclohexylmethoxy)methyl}-5-methylbenzoic acid hydrochloride, Japan Tobacco Inc., Tokyo, Japan) and tolbutamide (Wako Pure Chemical Industries, Osaka, Japan) in the oral glucose tolerance test (OGTT), each
compound was administered orally to 16-h-fasted rats. After 30 min, glucose solution (2 g/kg) was administered orally. Blood samples were obtained before administration of the compounds and glucose solution as well as 10, 30, 60, 120, and 180 min after glucose loading. Plasma glucose and insulin levels were measured as described above. The area under the curve (AUC [0–10]) for incremental plasma insulin levels (ΔINS) during IVGTT were calculated using the trapezoidal rule. To evaluate the insulin response to glucose during OGTT, the insulinogenic index (ΔINS/ΔGLU) was calculated using incremental plasma insulin and glucose levels for 0 to 10 min after glucose loading.

In the intravenous arginine tolerance test (IVATT), L-arginine solution (0.2 g/kg) was administered via the tail vein to 4-h-fasted rats. Blood samples were collected before (0 min), and 3 and 6 min after L-arginine loading. The area under the curve (AUC [0–6]) for incremental plasma insulin levels (ΔINS) during IVATT were calculated using the trapezoidal rule. Plasma insulin levels were measured as described above.

Measurements of insulin and glucagon content in pancreas

Acid/ethanol extraction was performed in accordance with the method of Kenny et al. [6]. Briefly, the pancreas was removed promptly and homogenized in a cold acid/ethanol mixture (75% ethanol, 23.5% distilled water, 1.5% 2 N hydrochloric acid) to extract insulin and glucagon. The levels of insulin and glucagon in the extract were measured with an ELISA kit (glucagon ELISA kit; Wako Pure Chemical Industries).

Immunohistochemistry evaluation of pancreatic islets

The pancreas samples were fixed in 10% formalin and embedded in paraffin. Serial sections were prepared and immunofluorescence staining for insulin and glucagon was performed by adding mouse anti-insulin and rabbit anti-glucagon (Nichirei, Tokyo, Japan) followed by anti-mouse Alexa 488 and anti-rabbit Alexa 594 (Invitrogen, CA, USA) were used to visualize insulin- and glucagon-staining. Images were recorded using fluorescence microscopy (Olympus, Co., Tokyo, Japan).

Islet isolation

Islets of Langerhans were isolated from rat pancreas by collagenase digestion. Rats were anaesthetized by sodium pentobarbital. The pancreas was distended by slowly injecting 1 mg/ml collagenase solution (Nitta Gelatin, Osaka, Japan) from the bile duct. The pancreas was removed and incubated in a CO₂ incubator (95% air and 5% CO₂) for approximately 20 min at 37°C. The digested pancreas was washed twice with Hank’s balanced salt solution (HBSS) containing 0.1 mg/ml kanamycin (Invitrogen) and centrifuged at 1,800 rpm for 30 s and the supernatant was discarded. The islets and contaminants were separated by Ficoll-Conray gradient centrifugation. The islets were suspended in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose) containing 10% fetal bovine serum and 0.1 mg/ml kanamycin, then incubated in a CO₂ incubator at 37°C.

Measurement of insulin secretion from pancreatic isolated islets

A 1.5 ml aliquot of DMEM (low glucose) containing 10% FBS and kanamycin was plated into each well of a 6-well culture plate. Isolated islets of approximately equal size were selected under a stereoscopic microscope and five islets were transferred to each well. Krebs-Ringer bicarbonate buffer containing 10 mM HEPES (KRKH) and 0.2% fatty acid-free bovine serum albumin (BSA) (equilibrated with 95% O₂ and 5% CO₂) were used for the insulin secretion assay. The islets were preincubated for 1 h in KRKH containing 3.3 mM glucose. Following preincubation, the islets were transferred to KRKH containing various stimulants and incubated for an additional 1 h. Glucose, tolbutamide, and glucagon-like peptide-1 (7–36) amide (tGLP-1) (Sigma-Aldrich, St. Louis, MO, USA), were used as stimulants. Tolbutamide was dissolved in dimethyl sulfoxide and diluted with KRKH to a final concentration of 1%. tGLP-1 was dissolved in Dulbecco’s phosphate buffered saline without calcium and magnesium and then diluted with KRKH. After incubation, the supernatant was collected and the concentration of insulin released was measured. After the insulin secretion assay, the isolated islets were harvested and the genomic DNA was purified using a DNeasy tissue kit (QIAGEN, Tokyo, Japan). The concentration of the purified DNA was measured using a
PicoGreen dsDNA quantitation kit (Invitrogen). The amount of insulin secretion was calculated as ng/μg islet DNA/h for each stimulant.

**Dipeptidyl peptidase IV inhibition measurement in vitro**

To evaluate the inhibitory effect of JTP-76209 on DPP IV, 25 μl of enzyme source (human or rat plasma), 15 μl of enzyme reaction solution (containing 25 mM HEPES, 140 mM NaCl, 80 mM MgCl₂, and 1% BSA, pH 7.8), 10 μl of JTP-76209, and 50 μl of substrate (100 μM Gly-Pro-4-methyl-coumaryl-7-amide (MCA); Peptide Institute, Osaka, Japan) were mixed in each well of a 96-well plate. The mixture was thoroughly stirred, then incubated at 37°C for 60 min. The fluorescence intensity at Ex 360 nm and Em 530 nm was measured using a fluorescence plate reader (CytoFluor II; Biosearch Technologies, Noverato, CA). The concentration of 7-amino-4-methylcoumarin amino acid (AMC) in the reaction solution was calculated from the AMC standard curve, and the DPP IV inhibitory activity (IC₅₀) of JTP-76209 was estimated. A Lineweaver Burk Plot was constructed to calculate the Ki value of JTP-76209.

**Human fibroblast activation protein α inhibition measurement in vitro**

A cDNA encoding human fibroblast activation protein α (FAP) was amplified by Nested-PCR from Human Ovary Marathon-Ready cDNA (Clontech, CA, USA). After the confirmation of its nucleotide sequence, the cDNA without an N-terminal region containing a transmembrane region was amplified by PCR and inserted into pFLAG-CMV-3 expression vector to create preprotrypsin leader sequence/FLAG-tag/FAP fusion protein. HEK293 cells were transfected with the vector using 293fectin and incubated for 48 h. The culture supernatant was applied to M2-agarose affinity chromatography and FLAG-tagged FAP protein was eluted from the column in the presence of 100 μg/ml FLAG peptide. FLAG peptide was removed by an Econo-Pac 10DG column (Bio-rad, CA, USA) from each fraction to get purified human FAP enzyme.

A 10-μl sample of JTP-76209, 70 μl of the reaction solution (containing 100 mM Tris-Cl and 100 mM NaCl, pH 7.8), 10 μl of synthetic substrate (5 mM Ala-Pro-7-amino-4-trifluoromethyl coumarin (AFC); Bachem, Switzerland), and 10 μl of human purified FAP enzyme were plated into each well of a 96-well plate. The mixture was thoroughly stirred, then incubated at 37°C for 60 min. The fluorescence intensity at Ex 360 nm and Em 530 nm was measured using a fluorescence plate reader (CytoFluor II; Biosearch Technologies, Noverato, CA). The concentration of AMC in the reaction solution was calculated from the AMC standard curve, and the FAP inhibitory activity (IC₅₀) of JTP-76209 was estimated. A Lineweaver Burk Plot was constructed to calculate the Ki value of JTP-76209.

**Statistical analysis**

All results are expressed as the mean ± standard error. Statistical analyses of the differences between the mean values were performed using the F test, followed by Student’s t-test or Aspin-Welch’s t-test. Differences were considered as significant at P<0.05.

**Results**

**Analysis of body weight and biochemical parameters**

Body weight of SDT rats showed a decreasing tendency from 16 weeks of age and were significantly decreased at 24 weeks of age (SD: 622 ± 23.9 g vs SDT: 520 ± 16.6 g) (Fig. 1A), as compared with that of control SD rats. Plasma insulin levels of SDT rats at 16 weeks of age showed no significant changes as compared with age-matched SD rats, but after 16 weeks the insulin levels decreased rapidly with age. At 24 weeks of age, plasma insulin levels of SDT rats were lower than those of SD rats (SD: 3.17 ± 0.33 ng/ml vs SDT: 1.43 ± 0.42 ng/ml) (Fig. 1B). While no significant change was noted in the plasma glucose levels of SDT rats at 16 weeks of age as compared with that of SD rats, the levels were significantly increased at 24 weeks of age (SD: 129 ± 3.8 mg/dl vs SDT: 612 ± 61.2 mg/dl) (Fig. 1C).

**Evaluation of insulin secretion in pancreatic isolated islets**

In the isolated islets of SDT rats at 8 weeks of age, insulin secretion in response to 3.3, 5.6, and 11.2 mM glucose was comparable to that in isolated islets of age-matched SD rats. Insulin secretion with 22.4 mM glucose was significantly increased in the islets of SDT rats.
as compared with that in the islets of SD rats (Fig. 2A). At 16 weeks of age, however, insulin secretion in response to 5.6, 11.2, and 22.4 mM glucose was significantly decreased in the islets of SDT rats as compared with that of SD rats (Fig. 2B). To compare insulin secretion in the islets of SD and SDT rats at 8 and 16 weeks of age, insulin secretion in response to 22.4 mM glucose relative to the amount of insulin secretion in response to 5.6 mM glucose in each age group was calculated. At 8 weeks of age, the mean insulin release in both SDT and SD rats was similar at 119- and 125-fold the amount secreted in response to 5.6 mM glucose, respectively, whereas at 16 weeks of age, the mean release in SDT rats decreased to half of that in SD rats (17- and 8-fold...
the amount secreted in response to 5.6 mM glucose for SD and SDT rats, respectively). Therefore, it was confirmed that glucose-stimulated insulin secretion (GSIS) decreased with age in SDT rats. It should be noted that pancreatic islets could not be isolated from SDT rats at 24 weeks of age.

**Immunohistochemistry of pancreatic islets**

In the islets of 8-week-old SDT rats, dense insulin immunostaining was observed in pancreatic β cell cytoplasm (green), whereas glucagon immunostaining of the α cells (red) appeared as a dense rim at the islet periphery, with similar images noted for age-matched SD rats (Fig. 3). In 16-week-old SDT rats, though, mixed composition of insulin- and glucagon-immunostained images as well as morphological changes in islets were confirmed. In 24-week-old SDT rats, few insulin-immunostained images were observed.

**Evaluation of insulin and glucagon content of the pancreas**

The insulin content of the pancreas of 8-week-old SDT rats was similar to that of age-matched SD rats. At 16 weeks of age, the insulin content of the pancreas of SDT rats decreased to approximately 68% that of SD rats (SD: 134 ±14 μg/pancreas vs SDT: 91 ±22 μg/pancreas). At 24 weeks of age, the insulin content of the pancreas of SDT rats decreased to approximately 4% that of SD rats (SD: 157 ± 7 μg/pancreas vs SDT: 7 ± 2 μg/pancreas) (Table 1). The glucagon content of the pancreas in both SDT and SD rats was similar at 8 weeks of age whereas the contents in SDT rats at 16 and 24 weeks of age showed decreases of approximately 56% (SD: 7.1 ± 0.6 μg/pancreas vs SDT: 4.0 ± 0.3 μg/pancreas) and 36% (SD: 6.4 ± 0.5 μg/pancreas vs SDT: 2.3 ± 0.5 μg/pancreas) (Table 1), respectively, as compared with SD rats. Based on these findings, the insulin and glucagon content of the pancreas in SDT rats decreased with age and the rate of decrease in insulin content, in particular, accelerated rapidly from 16 weeks of age.

**Evaluation of tolbutamide and tGLP-1-stimulated insulin release in pancreatic isolated islets**

In the islets of SDT rats, insulin release in response to 11.2 mM glucose decreased, as compared with age-matched SD rats. When islets of SDT rats were treated with 1 or 10 nM tGLP-1 in the presence of 11.2 mM glucose, an increase in the insulin release was observed, with the amount of insulin released following 1 nM tGLP-1 treatment being comparable to that in isolated
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islets of SD rats (SD: 119 ± 20 ng/µg islet DNA/h vs 1 nM tGLP-1 treated SDT: 112 ± 42 ng/µg islet DNA/h). In addition, a comparison of the mean insulin release (% of control) following treatment with 1 nM tGLP-1 showed that the release in SD and SDT rats was 259 and 262%, respectively (Fig. 4A). When islets of 16-week-old SDT rats were treated with 10 and 30 µM tolbutamide in the presence of 11.2 mM glucose, an increase in the insulin release was observed, with the amount of insulin released following treatment with 30 µM tolbutamide comparable to that in isolated islets of SD rats (SD: 83 ± 25 ng/µg islet DNA/h vs 30 µM tolbutamide treated SDT: 63 ± 41 ng/µg islet DNA/h). In addition, the mean insulin release (% of control) following treatment with 30 µM tolbutamide showed that the release in SD and SDT rats was 294 and 656%, respectively (Fig. 4B). Based on these findings, although GSIS decreased in 16-week-old SDT rats, insulin secretion in response to non-glucose stimulation (tGLP-1, tolbutamide) was comparable to that in SD rats.

Table 1. Changes of pancreatic insulin and glucagon content in SD and SDT rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body weight (g)</th>
<th>Pancreas weight (g)</th>
<th>Insulin µg/pancreas</th>
<th>Insulin µg/g tissue</th>
<th>Glucagon µg/pancreas</th>
<th>Glucagon µg/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 weeks of age</td>
<td>SD</td>
<td>6</td>
<td>320 ± 8</td>
<td>0.59 ± 0.03</td>
<td>100 ± 32</td>
<td>179 ± 62</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>SDT</td>
<td>6</td>
<td>319 ± 7</td>
<td>0.65 ± 0.05</td>
<td>102 ± 19</td>
<td>158 ± 26</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>16 weeks of age</td>
<td>SD</td>
<td>6</td>
<td>528 ± 11</td>
<td>0.83 ± 0.05</td>
<td>134 ± 14</td>
<td>161 ± 13</td>
<td>7.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>SDT</td>
<td>6</td>
<td>502 ± 6</td>
<td>0.68 ± 0.06</td>
<td>91 ± 22</td>
<td>135 ± 29</td>
<td>4.0 ± 0.3**</td>
</tr>
<tr>
<td>24 weeks of age</td>
<td>SD</td>
<td>6</td>
<td>629 ± 24</td>
<td>1.14 ± 0.15</td>
<td>157 ± 7</td>
<td>152 ± 23</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>SDT</td>
<td>6</td>
<td>513 ± 16**</td>
<td>0.67 ± 0.05*</td>
<td>7 ± 2**</td>
<td>10 ± 2**</td>
<td>2.3 ± 0.5**</td>
</tr>
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</table>

SDT rats exhibited significantly decreased pancreatic insulin content as well as decreased glucagon content at 24 weeks of age. Each value represents mean ± SEM. (n=6). *P<0.05, **P<0.01; significantly different from age-matched SD rats.

Fig. 4. Evaluation of tGLP-1 (A) and tolbutamide-stimulated insulin (B) secretion in pancreatic islets isolated from SD (open column) and SDT rats (closed column) at 16 weeks of age. Insulin secretion in islets of 16-week-old SDT rats was restored by tolbutamide or tGLP-1 stimulation. Each value represents mean ± SEM. (n=4). NS, not significant; different from 11.2 mM glucose treated insulin secretion of islets isolated from SD rats.
Intravenous glucose tolerance test (IVGTT) and intravenous arginine tolerance test (IVATT)

In 8-week-old SDT rats, plasma insulin levels before (0 min), as well as 5 and 10 min after glucose loading, were lower than those of age-matched SD rats, whereas mean plasma insulin levels 5 min after glucose loading were increased 2.56 ng/ml, compared to 1.02 ng/ml at baseline (Fig. 5A). Moreover, Δ insulin AUC for 0 to 10 min (AUC [0–10]) for SDT rats was comparable to that for SD rats (Fig. 5C). In 16-week-old SDT rats, no increase in plasma insulin levels after glucose loading was noted (Figs. 5B and 5D). This marked decrease in GSIS in 16-week-old SDT rats agreed with a decrease in GSIS was observed in islets isolated from SDT rats.

For 8-week-old and 16-week-old SDT rats, the area under the curve (AUC [0–6]) of incremental plasma insulin levels (ΔINS) during IVATT were not different from the AUC of age-matched SD rats (8-week-old SD: 10.58 ± 3.56 ng/ml-min vs 8-week-old SDT: 9.35 ± 1.63 ng/ml-min; 16-week-old SD: 3.52 ± 2.43 ng/ml-min vs 16-week-old SDT: 6.66 ± 4.78 ng/ml-min).

Evaluation of DPP IV inhibitor and tolbutamide on SDT rats in the oral glucose tolerance test

JTP-76209 was used as the DPP IV inhibitor (Fig. 6A). JTP-76209 exhibited a DPP IV inhibitory activity (IC$_{50}$) in humans and rats of 6 nM (K$_{i}$=2 nM) and 31 nM (K$_{i}$=22 nM), respectively. In order to evaluate the selectivity of JTP-76209 to DPP IV enzyme, human FAP inhibition was assessed. The IC$_{50}$ of this enzyme was shown to be

Fig. 5. Evaluation of insulin secretion after glucose loading (A, B; open circle: SD rat, closed circle: SDT rat) in SD and SDT rats. The additional insulin secretion after glucose loading in SDT rats was decreased at 16 weeks of age (B, D). Each value represents mean ± SEM. (n=6). *P<0.05, **P<0.01; significantly different from the SD rats.
10 μM, thereby confirming a 1,500-fold or more difference in inhibition compared to that of the DPP IV enzyme. As a non-specific test, the effects of 30 μM JTP-76209 on the different kinds of receptors (n=23) and enzymes (n=7) were assessed. The receptor binding activity and enzyme inhibitory activity, however, could not be calculated (data not shown). Therefore, these results showed that JTP-76209 is a DPP IV selective
inhibitor. The improvement effects of JTP-76209 (10 mg/kg) and tolbutamide (100 mg/kg) on insulin secretion and glucose tolerance after glucose loading were examined in SDT rats. Both compounds increased insulin release at 10 min after glucose loading, with an improvement in glucose tolerance at 30 and 60 min after glucose loading (Figs. 6B and 6C). The insulinogenic index was calculated from plasma insulin and glucose levels which were derived from plasma insulin and glucose levels before (0 min), and 10 min after glucose loading. The insulinogenic index of 16-week-old SDT rats was lower than that of SD rats (SD: 491 × 10⁻⁴ vs SDT: 82 × 10⁻⁴) (Fig. 6D). Moreover, the insulinogenic index of SDT rats following administration of JTP-76209 was significantly increased, and that of tolbutamide showed an increasing tendency, compared to the control group (Fig. 6D).

**Discussion**

The SDT rat which develops hypoinsulinemia and hyperglycemia with age is a spontaneous model of non-obese type 2 diabetes mellitus [11, 24]. The mechanism of onset of diabetes mellitus in SDT rats has been unclear. Shinohara *et al.*, however, reported that lesions, including bleeding and inflammatory cell infiltration, were observed in the pancreatic islets of pre-diabetic SDT rats [24]. Therefore, changes in pancreatic function may be an important factor in the onset of diabetes mellitus in SDT rats. In the present study of pancreatic function of SDT rats, *in vitro* and *in vivo* evaluations of age-related changes in function were performed in order to examine the changes in pancreatic function leading to the onset of diabetes mellitus in SDT rats.

An analysis of body weights and biochemical parameters revealed no clear changes in SDT rats at 16 weeks of age, whereas weight loss, elevated plasma glucose level, and decreased plasma insulin level were observed at 24 weeks of age (Fig. 1). These changes coincided with those reported by Masuyama *et al.* [11, 24]. In an *in vitro* assay using islets, on the other hand, a marked decrease in GSIS was observed in SDT rats at 16 weeks of age (Fig. 2), with abnormalities in insulin secretion noted prior to the onset of diabetes mellitus at 24 weeks of age. Regarding the etiology of type 2 diabetes mellitus in humans and the question of whether impaired insulin secretion or insulin resistance is the initial symptom, most epidemiological studies have reported that decreased insulin secretion precedes the onset of type 2 diabetes [8, 9]. The present study also confirmed a decrease in GSIS prior to the onset of diabetes mellitus in SDT rats. Therefore, the SDT rat is a useful animal model for the analysis of the etiology of diabetes mellitus in humans.

Changes in pancreatic function and morphology due to metabolic abnormalities have been widely investigated in pathological models. While current models of spontaneous diabetes mellitus typically have characteristics of obesity and hyperinsulinemia (e.g., KK-*A* mouse [22], ob/ob mice [3], ZF rats [23], ZDF rats [26], etc.) as well as exhibiting hyperplasia and hypertrophy of islets, it has been reported that, in SDT rats, the pancreatic β cell number decreases with age. Therefore, it has been suggested that the SDT rat may be a useful, new model of non-obese diabetes mellitus. In SDT rats, decreased β cell number, islet deformation (Fig. 3), and decreased insulin content (Table 1) are observed. Similar changes, however, are also seen in the GK rat model of non-obese diabetes mellitus. The GK rat is an animal model which exhibits hepatic insulin resistance in addition to functional and morphological changes in the pancreas, without obesity. In isolated islets of GK rats, GSIS is significantly decreased; thus, the first phase of GSIS is suppressed [7]. On the other hand, since the insulin secretory response to non-glucose (e.g., arginine, glibenclamide, α-ketoisocaproic acid) stimulation is well preserved in GK rats [15], the impaired glucose tolerance of GK rats is considered to be the primary cause of impaired insulin secretion in response to glucose stimulation in pancreatic β cells. In the present study, a decrease in GSIS was observed in isolated islets of SDT rats, but non-glucose (tolbutamide, tGLP-1)-stimulated insulin secretion was relatively preserved (Fig. 4). We confirmed the expression of sulfonylurea receptor-1 (SUR1) and tGLP-1 receptor mRNA in isolated islet from SDT rats at 16 weeks of age (data not shown). An *in vivo* study showed that arginine-stimulated insulin secretion in SDT rats at 8 and 16 weeks of age was not different from that of SD rats, whereas glucose-stimulated insulin secretion at 16 weeks of age was markedly decreased
(Fig. 5). Therefore, it was shown that changes in pancreatic function in the pre-diabetic SDT rat are similar to those in GK rats. In contrast, in models of spontaneous diabetes with hyperplasia and hypertrophy of pancreatic islets, decreased insulin secretion after glucose loading has been reported. ZF rats have a leptin receptor signaling defect that leads to hyperphagia and obesity [1]. In addition, increases in non-fasting and fasting plasma insulin levels are observed as peripheral insulin resistance progresses. Christopher et al. [4] have reported that the DNA and insulin content of pancreatic islets in ZF rats at 17 to 18 weeks of age are approximately 3-fold higher than those in Zucker Lean (ZL) rats and that the pancreatic $\beta$ cell number in ZF rats is also higher. They also reported the development of impaired GSIS, based on the observation that insulin secretory response in the presence of a high glucose (16 mM) in isolated islets of ZF rats is half that of ZL rats, whereas the insulin secretory response with 300 $\mu$M tolbutamide is the same as that in ZL rats [4]. It is possible that chronically elevated plasma free fatty acids (FFA) due to peripheral insulin resistance may account for this impairment but the details are unclear.

It has previously been reported that chronic hyperglycemia and chronically elevated FFA lead to pancreatic $\beta$ cell dysfunction as a manifestation of glucotoxicity and lipotoxicity [17–19]. The mechanism of $\beta$ cell failure is mediated by the accumulation of triglycerides in $\beta$ cells resulting from chronically elevated FFA, which cause ceramide and iNOS-activated NO production, etc. and, finally, apoptosis of $\beta$ cells [28, 30]. While there have been no detailed reports on peripheral insulin resistance in 16-week-old SDT rats, the present study calculated the HOMA-IR (homeostasis model assessment of insulin resistance) index (fasting glucose concentration $\times$ fasting insulin concentration/22.5) [16] and showed that the index tends to be higher for SDT rats than for SD rats (SD: 3.7 ± 0.8 vs SDT: 6.7 ± 1.8). Further studies are needed to examine changes in insulin resistance in SDT rats with age using the insulin tolerance test or the hyperinsulinemic-euglycemic glucose clamp and to investigate the relationship with changes in pancreatic function.

The present study confirmed the effects of the DPP IV selective inhibitor, JTP-76209 (Fig. 6A), and the sulfonylurea (SU) agent, tolbutamide, in 16-week-old SDT rats which exhibit glucose intolerance associated with impaired insulin secretion. It was shown that both compounds improve glucose tolerance by enhancing insulin secretion (Fig. 6). This effect on insulin impairment has also been reported in GK and neonatal streptozotocin (nSTZ)-induced rats (in the case of SU agents) [12, 13] as well as ZDF and ZF rats (in the case of DPP IV inhibitors) [2, 25]. The results of our in vivo study on the effects agreed with the finding in isolated islets of SDT rats that insulin secretion is enhanced by tGLP-1 or tolbutamide stimulation (Fig. 4). These results suggest that the SDT rat will be a useful model for the development of antidiabetic agents.

Our study showed that GSIS in pre-diabetic SDT rats is impaired from at least 16 weeks of age and that DPP IV inhibitor and SU agent ameliorate impaired insulin secretion. We conclude that the SDT rat is a useful new model of non-obese impaired insulin secretion for the analysis of the onset of type 2 diabetes and the development of antidiabetic agents.

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References
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