Protective Effect of Ipragliflozin on Pancreatic Islet Cells in Obese Type 2 Diabetic db/db Mice

Toshiyuki Takasu* and Shoji Takakura

Tsukuba Research Center, Drug Discovery Research, Astellas Pharma Inc.; 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan.

Received December 15, 2017; accepted February 13, 2018

Ipragliflozin is a selective sodium glucose cotransporter 2 (SGLT2) inhibitor that increases urinary glucose excretion and subsequently improves hyperglycemia in patients with type 2 diabetes mellitus (T2DM). To assess the beneficial effect of ipragliflozin on the mass and function of pancreatic β-cells under diabetic conditions, obese T2DM db/db mice were treated with ipragliflozin for 5 weeks. Glucose and lipid metabolism parameters, pathological changes in pancreatic islet cells and insulin content were evaluated. Pathological examination of pancreatic islet cells comprised measuring the ratios of insulin- and glucagon-positive cells and levels of oxidative stress markers. Hemoglobin A1c, plasma glucose, non-esterified fatty acid and triglyceride levels in ipragliflozin-treated groups were reduced compared to the diabetic control (DM-control) group. Histopathological examination of pancreatic islet cells revealed strong insulin staining and reduced glucagon staining in the ipragliflozin 10 mg/kg-treated group compared with the DM-control group. The ratio of α- to β-cell mass was lower in the ipragliflozin 10 mg/kg-treated group than the DM-control group and was similar to that of the non-diabetic control group. The density of immunostaining for 4-hydroxy-2-nonenal, an oxidative stress marker, in pancreatic islets was significantly lower in the ipragliflozin 10 mg/kg-treated group than the DM-control group. Pancreatic insulin content tended to be higher in the ipragliflozin-treated groups than the DM-control group. Our findings demonstrate the benefit of ipragliflozin treatment in improving glucolipotoxicity and reducing oxidative stress in pancreatic islet cells. Treatment with ipragliflozin may protect against the progressive loss of islet β-cells in patients with T2DM.

Key words: ipragliflozin; sodium glucose cotransporter 2 inhibitor; anti-diabetic drug; pancreatic islet cell; type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a chronic and progressive disease. The main characteristics of T2DM are insulin resistance in insulin target tissues and insufficient insulin secretion from pancreatic β-cells.1,2) Insulin resistance develops as a result of overeating, physical inactivity and as a consequence of obesity and fat accumulation in the body caused by excessive insulin secretion from pancreatic β-cells.3) In the prediabetic state, a sufficient amount of insulin is secreted from β-cells to compensate for insulin resistance.4) Subsequently, larger adipocytes accumulate in adipose tissue and secrete large amounts of free fatty acids (FFAs) and inflammatory cytokines, which leads to a deterioration in β-cell function. This process is well known as β-cell lipotoxicity.5) After the collapse of compensatory insulin secretion mechanisms, β-cells are chronically exposed to hyperglycemia, leading to a gradual deterioration in function and decrease in β-cell mass. These phenomena are well known as β-cell glucose toxicity.6–8) Several studies have suggested that increased FFA concentration and hyperglycemia induce adverse modifications to cell components such as lipids, proteins and DNA in pancreatic β-cells through the increased formation of reactive oxygen species (ROS), which leads to oxidative stress.9–12) Due to relatively low expression of antioxidant enzymes, such as catalase, selenium-dependent glutathione peroxidase 1 (GPX1), and Cu, Zn-superoxide dismutase 1 (SOD1), β-cells are highly sensitive to ROS.13) Persistent hyperglycemia results in constitutive stimulation of β-cells, and therefore increases insulin biosynthesis, which can induce endoplasmic reticulum (ER) stress.14) Both oxidative stress and ER stress are involved in β-cell dysfunction in T2DM.15–16)

Sodium glucose cotransporter 2 (SGLT2) inhibitors have recently been developed as antidiabetic drugs.17,18) SGLT2, a low-affinity and high-capacity glucose transporter, is expressed specifically on the luminal surface of cells in the S1 segment of the proximal tubule19) and reabsorbs approximately 90% of glucose in the glomerular filtrate in normal glucose-tolerant individuals.20) SGLT2 inhibitors prevent this reabsorption, thereby increasing urinary glucose excretion and subsequently decreasing blood glucose levels. Because SGLT2 inhibitor action is not dependent on insulin secretion, SGLT2 inhibitors are expected to reduce the insulin secretory demand, and therefore preserve pancreatic β-cell function in patients with T2DM.

Using animal models of T2DM, several studies have demonstrated the preservation of pancreatic β-cells by SGLT2 inhibitors.20–24) Tofogliflozin, dapagliflozin, empagliflozin and luseogliflozin lower blood or plasma glucose levels and increase plasma insulin levels, and preserve pancreatic β-cell function in obese T2DM db/db mice.20–23) Empagliflozin and luseogliflozin increase the expression levels of insulin-related genes and a proliferation marker protein, and reduce glucose toxicity and an apoptosis maker protein in islets of obese T2DM db/db mice.22,23) Empagliflozin has antihyperglycemic effects in Zucker diabetic fatty rats, and slows the degradation of pancreatic β-cell function and β-cell mass.24) However, the mechanism underlying the positive effects of SGLT2 inhibitors on pancreatic β-cell preservation has not been fully investigated. Although the attenuation of oxidative stress in pancreatic islet cells by SGLT2 inhibitors is a possible mechanism of action,25–28) studies on this subject are limited. Upregulation of
α-cell mass has recently been reported as a possible primary driving force of hyperglycemia in T2DM, however, to our knowledge, the effect of SGLT2 inhibitors on α-cell mass or number has not been investigated.

In this study, we investigated the effect of the selective SGLT2 inhibitor ipragliflozin on oxidative stress and pathological changes, including the α- to β-cell ratio in pancreatic islet cells, in obese T2DM db/db mice. We also examined its effects on plasma parameters for glucose and lipid metabolism, and pancreatic insulin content.

MATERIALS AND METHODS

Drugs Ipragliflozin L-proline was synthesized at Astellas Pharma Inc. (Ibaraki, Japan). The compound was suspended in 0.5% methylcellulose solution (0.5% MC) as a vehicle, and administered by oral gavage at 10 mL/kg of body weight. The dose of ipragliflozin is expressed as a proline-free substance.

Animals and Diets All animal experiments were conducted in the Kumamoto Laboratory, LSI Medience Corporation, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Male BKS. Cg-+Lepr+/+Lepr+/+Jcl (hereafter: db/db) mice and male BKS. Cg-m +/+ Lepr+/+Jcl (hereafter: db/m+) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were housed under specific pathogen-free conditions with controlled temperature, humidity, and light (12:12h light–dark cycle). Mice were fed a standard laboratory chow (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Tsukuba Research Center of Astellas Pharma Inc., which is accredited by the AAALAC International.

Study Design At age 8 weeks, the male db/db mice were allocated into 3 groups: (1) control (n=12, diabetic (DM)-control), administered vehicle; (2) ipragliflozin 1 mg/kg/d (n=12); and (3) ipragliflozin 10 mg/kg/d (n=12), so that each group had similar mean body weights and plasma glucose, plasma insulin and hemoglobin A1c (HbA1c) levels. Male db/m+ mice administered vehicle served as the non-diabetic (ND)-control group (n=12). Ipragliflozin or vehicle was administered orally once daily in the evening for 5 weeks. Body weight and residual food were measured once a week and twice a week, respectively, using a digital balance. The final administration (Day 28) was followed by a 2-d withdrawal period, and an oral glucose tolerance test (OGTT) was performed the day after completion of withdrawal (Day 31). After a fast of 16h, blood samples were collected from the tail vein of mice before and 15, 30, 60, 120, and 180 min after glucose administration (2 g/kg/10 mL). Plasma glucose and insulin levels were measured, and the area under the blood concentration–time curve (AUC) levels of these parameters were evaluated. After the OGTT, administration of vehicle or ipragliflozin was resumed for 1 week. The next morning (Day 38), animals were anesthetized with inhalation of a gas mixture (2% isoflurane, 65% N2O and 35% O2), and a blood sample was collected from the inferior vena cava using a heparinized syringe under nonfainting conditions, and the pancreas was isolated. The blood sample was centrifuged at 4°C and 1890 x g for 10 min, and plasma was obtained for the measurement of plasma glucose, insulin, non-esterified fatty acid (NEFA) and triglyceride (TG) concentrations.

Blood Biochemical Assays Plasma glucose levels were measured using Glucose CII-Test reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma insulin levels were measured using the Ultra-Sensitive Mouse Insulin enzyme-linked immunosorbent assay (ELISA) Kit (Moriga Institute of Biological Science, Inc., Kanagawa, Japan). Plasma NEFA and TG concentrations were measured spectrophotometrically using a Hitachi 7170 auto analyzer (Hitachi High Technologies Co., Tokyo, Japan). HbA1c levels were quantified using a DCA2000 System (Siemens K.K., Tokyo, Japan).

Pancreas Collection and Sampling After blood sampling, the animals were euthanized by exsanguination. The pancreas was extracted and weighed. The pancreases of odd-numbered animals were fixed with 10% neutral buffer formalin solution, while the pancreases of even-numbered animals were frozen in liquid nitrogen and stored in a deep freezer until insulin measurements.

Measurement of Insulin Content in the Pancreas Insulin levels in frozen pancreases were measured according to the method described by Harrity et al. A volume of 5 mL of HCl–ethanol extract (HCl: 0.15 mol/L, ethanol: 75%, distilled water: 23.5%) was added to each frozen pancreas and homogenized. The pancreas was subsequently extracted for more than 16 h at 3 to 4°C. The mixture was centrifuged at 40°C and 3000 rpm for 30 min, and the supernatant was stored in a deep freezer until measurement. The insulin content in the extracted supernatant solution was measured using the Ultra-Sensitive Mouse Insulin ELISA Kit, and was expressed as ng/g of pancreas.

Preparation of Pancreas Pathological Specimens and Histopathological Findings The pancreases were fixed with 10% neutral buffer formalin solution, embedded in paraffin and sectioned at 2–3 μm thickness according to standard procedures. The pancreas sections were immunostained using rabbit anti-insulin polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, U.S.A.), rabbit anti-glucagon polyclonal antibody (Nichirei Biosciences Inc., Tokyo, Japan), mouse anti-proliferative cell number antigen (PCNA) monoclonal antibody (Nichirei Biosciences Inc., mouse anti-4-hydroxy-2-nonenal (4-HNE) modified protein monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan), and anti-rabbit C/EBP homologous protein (CHOP) polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, U.S.A.). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL), a method for detecting apoptotic DNA fragmentation, was performed according to the manufacturer’s instructions (ApopTag® Peroxidase In Situ Apoptosis Detection Kit S7100, Merck KGaA, Darmstadt, Germany). Histofine® simple stain mouse MAX-PO (R) (Nichirei Biosciences Inc.) was used as a secondary antibody. The total number of islet cells and the number of cells positive for each antibody in the islets of Langerhans were counted and used to calculate the positive cell ratio. All or up to 50 islets/mouse were analyzed and expressed as a percentage of the total number of islet cells.

Statistical Analysis Results are expressed as mean±standard error of the mean (S.E.M.). Differences between the ND-control group and DM-control group were evaluated using Student’s t-test. Differences between the DM-control group and ipragliflozin-treated groups were analyzed...
using Dunnett’s multiple comparisons test. For each test, statistical significance was defined by a p value less than 0.05. All data analyses were conducted using SAS statistical software (version 9.1.3, SAS Institute Japan, Ltd., Tokyo, Japan).

RESULTS

Body Weight and Total Food Intake The body weights of the 3 groups of obese T2DM db/db mice at the beginning of the study did not significantly differ (DM-control: 35.7±0.5 g, ipragliflozin 1 mg/kg: 34.6±0.6 g, ipragliflozin 10 mg/kg: 34.2±0.5 g), and were all higher than that of the ND-control group (26.5±0.5 g). After treatment for 38 d, body weight gain in the ipragliflozin 10 mg/kg-treated group (8.0±0.7 g) was significantly higher than that in the DM-control group (4.5±0.7 g) (Table 1). Total food intake in the DM-control group (244.7±5.3 g) was significantly higher than that in the ND-control group (131.0±2.2 g). Ipragliflozin did not affect the amount of total food intake (Table 1).

Blood Biochemical Assays and Pancreatic Insulin Content HbA1c and non-fasting plasma glucose levels at age 7 weeks, before starting the treatment, and age 13 weeks, on Day 38 of treatment, were measured (Fig. 1). At age 7 weeks, mean HbA1c level was 3.4% in the ND-control group but ranged from 3.7 to 3.8% in the DM-control and ipragliflozin-treated groups. Mean HbA1c level in the DM-control group was significantly higher than that in the ND-control group. At age 13 weeks, HbA1c level in the ND-control group was virtually unchanged from that at age 7 weeks, whereas HbA1c level in the DM-control group (8.8±0.2%) was increased and significantly higher than that in the ND-control group (3.5±0.0%). The HbA1c level in the ipragliflozin-treated groups was significantly lower than that of the DM-control group (Fig. 1). Mean non-fasting plasma glucose level in the DM-control group was significantly higher than that in the ND-control group at age 7 weeks and 13 weeks. Treatment with ipragliflozin significantly reduced plasma glucose levels (Fig. 1). Plasma NEFA and TG levels in the DM-control group were significantly higher than those in the ND-control group after the 38-d treatment. Treatment with ipragliflozin significantly reduced plasma NEFA and TG levels (Fig. 2).

Following a 2-d withdrawal after 28 d of treatment with ipragliflozin, an OGTT was subsequently conducted to evaluate the effect of ipragliflozin on glucose tolerance and insulin sensitivity. The 2-d withdrawal was considered to be sufficient for drug washout. The OGTT demonstrated significant increases in the AUC of plasma insulin levels until 180 min after oral glucose loading in the ipragliflozin 10 mg/kg-treated group, and decreases in the AUC of plasma glucose levels in the ipragliflozin-treated groups (Fig. 3). Insulin content of pancreatic islets from the DM-control group was significantly reduced compared to the ND-control group. The content tended to be higher (p=0.067) in the ipragliflozin 10 mg/kg-treated group than in the DM-control group (Fig. 4).

Histological Findings Immunostaining with antibodies against insulin, glucagon, 4-HNE, PCNA, CHOP and TUNEL was conducted to investigate the protective effect of ipragliflozin on pancreatic β-cells (Figs. 5 and 6, Table 2). In the DM-control group, the insulin-positive cell (β-cell) ratio in pancreatic islet cells was significantly lower, while the glucagon-positive cell (α-cell) ratio was significantly higher than those in the ND-control group. Treatment with ipragliflozin 10 mg/kg significantly increased the insulin-positive cell ratio and decreased the glucagon-positive cell ratio compared to that in the DM-control group (Figs. 5A–D). The ratio of α-cells to β-cells in the DM-control group was significantly higher than that in the ND-control group. Treatment with ipragliflozin 10 mg/kg significantly decreased the ratio of α-cells to β-cells compared to the DM-control group (Fig. 5E). These results suggest that ipragliflozin treatment increased β-cell mass and reduced α-cell mass in obese T2DM db/db mice.

In the DM-control group, the 4-HNE-positive cell ratio in pancreatic islet cells was significantly higher than that in the ND-control group. Treatment with ipragliflozin 10 mg/kg significantly decreased the 4-HNE-positive cell ratio compared to the DM-control group (Fig. 6). The PCNA-, CHOP-, and TUNEL-positive cell ratios in pancreatic islet cells were significantly higher in the DM-control group than in the ND-con-
loading and improved glucose intolerance compared to the treated group showed increased insulin secretion after glucose pancreatic β-cells. In the OGTT, the ipragliflozin 10 mg/kg-tissue insulin sensitivity, 17,32) β-cells and urinary glucose excretion. As their mechanism of action is control; DM-control, diabetic control. p DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test (*p < 0.05 and **p < 0.01). Differences between the DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test (***p < 0.001). ND-control, non-diabetic control; DM, diabetes mellitus.

**Table 1. Effect of Ipragliflozin on Body Weight, Body Weight Gain and Total Food Intake in Obese T2DM db/db Mice**

|                | ND Control (n=12) | Ipragliflozin 1 mg/kg (n=12) | Ipragliflozin 10 mg/kg (n=12) |
|----------------|------------------|-----------------------------|-------------------------------|
| **Body weight (g)** |                  |                             |                               |
| Day 1          | 26.5±0.5         | 35.7±0.5*                   | 34.6±0.6                      |
| Day 38         | 29.0±0.5         | 40.2±0.9*                   | 39.9±1.0                      |
| **Body weight gain (g)** |            |                             |                               |
| Day 38         | 2.5±0.3          | 4.5±0.7*                    | 5.4±0.9                       |
| **Total food intake (g)** |            |                             |                               |
| Day 38         | 131.0±2.2        | 244.7±5.3*                  | 228.4±7.3                    |

Ipragliflozin was orally administered to diabetic db/db mice once daily for 38 d, starting at age 8 weeks. Values are expressed as mean±S.E.M. Differences between the ND-control group and DM-control group were determined using Student’s t-test (p < 0.05 and **p < 0.01). Differences between the DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test (***p < 0.001). ND-control, non-diabetic control; DM, diabetes mellitus.

**Fig. 2. Effect of Ipragliflozin L-Proline on Plasma Non-esterified Free Fatty Acid (NEFA) and Triglyceride (TG) Levels in Obese T2DM db/db Mice**

Following 38 d of treatment with ipragliflozin, plasma NEFA and TG concentrations were measured. (A) Plasma NEFA and (B) plasma TG. Values indicate mean±S.E.M. of 12 animals. Differences between the ND-control group and DM-control group were determined using Student’s t-test (p < 0.01). Differences between the DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test (***p < 0.001). ND-control, non-diabetic control; DM-control, diabetic control.

trol group. Treatment with ipragliflozin did not significantly affect these ratios (Table 2).

**DISCUSSION**

SGLT2 inhibitors reduce plasma glucose levels by inducing urinary glucose excretion. As their mechanism of action is independent of insulin secretion from pancreatic β-cells and tissue insulin sensitivity.17,32) SGLT2 inhibitors are expected to contribute to the preservation of pancreatic β-cell mass and function in patients with T2DM. In this study, we investigated the protective effect of the selective SGLT2 inhibitor ipragliflozin on pancreatic islet cells in obese T2DM db/db mice. Ipragliflozin reportedly increases urinary glucose excretion, improves hyperglycemia and prevents diabetic microvascular complications in T2DM mouse and rat models,33-35) and decreases blood glucose excursion after oral glucose loading in normal mice.36,37) In the present study, we found that HbA1c, plasma glucose, NEFA and TG levels were significantly decreased in ipragliflozin-treated groups compared to the DM-control group. Ipragliflozin also deceased the ratio of 4-HNE-positive cells in pancreatic islet cells, a marker for oxidative injury. Ipragliflozin increased the ratio of insulin-positive cells (β-cells) and tended to increase the pancreatic insulin content, but decreased the ratio of glucagon-positive cells (α-cells) in pancreatic islet cells. In the OGTT, the ipragliflozin 10 mg/kg-treated group showed increased insulin secretion after glucose loading and improved glucose intolerance compared to the DM-control group. Taken together, our findings suggest that the amelioration of glucolipotoxicity by ipragliflozin contributes to reducing oxidative stress and preserving β-cells, pancreatic insulin content and pancreatic β-cell function in obese T2DM db/db mice. Interestingly, an improvement in the ratio of α-cells to β-cells was also observed in ipragliflozin-treated groups.

Other SGLT2 inhibitors, including tofogliflozin, dapagliflozin, empagliflozin and luseogliflozin, have been shown to ameliorate hyperglycemia and preserve pancreatic β-cell function in obese T2DM db/db mice.20-23) Luseogliflozin increases β-cell proliferation and decreases β-cell apoptosis in pancreas tissue, which are accompanied by increased expression of insulin-related genes such as insulin 1, insulin 2, MafA, PD4-1 and GLUT2 in isolated islet cells from control mice and treated obese T2DM db/db mice.22,23) Luseogliflozin and empagliflozin increase Ki67-positive β-cells and reduce TUNEL-positive β-cells in the islets of obese T2DM db/db mice.22,23) These results suggest that treatment with SGLT2 inhibitors preserves β-cells. In the present study, we further investigated the effect of an SGLT2 inhibitor on pancreatic islet cells using immunohistochemical staining. We found that the ratios of 4-HNE+, CHOP+, PCNA- and TUNEL-positive cells in the DM-control group were significantly higher than those in the ND-control group. These results indicate an acceleration of oxidative stress, ER stress and apoptosis, and proliferative changes in pancreatic islet cells. Treatment with ipragliflozin significantly reduced the ratio of 4-HNE-
positive cells, suggesting an attenuation of oxidative stress. Ipragliflozin-associated decreases in plasma glucose, TG, and NEFA levels might contribute to the attenuation of oxidative stress by reducing glucolipotoxicity. Ipragliflozin reportedly reduces oxidative stress markers such as urine 8-hydroxy-2'-deoxyguanosine (8-OHdG), plasma thiobarbituric acid reactive substances (TBARS) and protein carbonyl levels in diabetic mice.37,38) In the present study, ipragliflozin-treated mice did not exhibit significantly reduced CHOP- or TUNEL-positive cells compared to the DM-control group. It is possible however that the very low frequency of CHOP- and TUNEL-positive cells in the DM-control group may have affected the evaluation of the effect of ipragliflozin on these parameters. Additionally, inflammatory cytokines reportedly contribute to the progressive loss of β-cell function and mass.25,39) We previously reported that, in addition to improving glycemic control in mouse models of diabetes, ipragliflozin reduces plasma inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemotactic protein-1 (MCP-1).38,40) Such an effect by ipragliflozin on inflammatory cytokines might contribute to its beneficial effects on pancreatic β-cells observed in the present study.

An imbalance in α-cell mass and β-cell mass has been reported in patients with type 2 diabetes and animal models.
Fig. 5. Effect of Ipragliflozin t-Proline on Pancreatic Islet Morphology, Insulin, Glucagon, and the Ratio of α-Cells to β-Cells in Pancreatic Islets of Obese T2DM db/db Mice

Following 38 d of treatment with ipragliflozin, the pancreas was dissected and pancreatic islet morphology was analyzed using immunohistochemical staining. Representative immunohistochemical staining for (A) insulin and (B) glucagon in pancreatic islet tissue sections. Scale bars: 20 μm. (C) Ratios of insulin-positive cells to all islet cells, (D) glucagon-positive cells to all islet cells and (E) α- to β-cells after the 38-d treatment with ipragliflozin. Values indicate mean±S.E.M. of 6 animals. Differences between the ND-control group and DM-control group were determined using Student’s t-test (**p<0.01). Differences between the DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test (##p<0.01). ND-control, non-diabetic control; DM-control, diabetic control.
Ipragliflozin was orally administered to diabetic db/db mice once daily for 38 d, starting at age 8 weeks. Values are expressed as mean±S.E.M. Differences between the ND-control group and DM-control group were determined using Student’s t-test (**p<0.01). Differences between the DM-control group and each ipragliflozin-treated group were assessed using Dunnett’s multiple comparisons test. ND-control, non-diabetic control; DM, diabetes mellitus; PCNA, proliferating cell nuclear antigen; CHOP, C/EBP homologous protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Table 2. Effect of Ipragliflozin on the Ratio of Positively-Labeled Cells in the Pancreatic Islet (by Histopathological Immunostaining) in Obese T2DM db/db Mice

| Positive cells (%) | ND Control (n=6) | Control (n=6) | Ipragliflozin 1 mg/kg (n=6) | Ipragliflozin 10 mg/kg (n=6) |
|--------------------|------------------|---------------|-----------------------------|-------------------------------|
| PCNA               | 2.57±0.31**      | 2.44±0.51     | 3.31±0.40                   |
| CHOP               | 0.56±0.10**      | 0.46±0.16     | 0.47±0.09                   |
| TUNEL              | 0.26±0.03**      | 0.14±0.06     | 0.22±0.11                   |

Of T2DM.39) This imbalance has also been suggested as a mechanism contributing to a decreased insulin to glucagon ratio,40) and thus, may be a primary driving force for hyperglycemia.41) Wang et al. reported that the ratio of α-cell to β-cell mass increased with age in obese T2DM db/db mice.39) Liu et al. also reported that α-cell number in pancreatic islets and plasma glucagon levels increased but plasma insulin levels decreased with diabetes progression.39) We showed similar results in DM-control mice. In particular, treatment with ipragliflozin increased insulin-positive cells but decreased glucagon-positive cells, thereby decreasing the ratio of α-cells to β-cells. To our knowledge, this is the first study to report that a SGLT2 inhibitor improved the imbalance in the ratio of α-cell to β-cell mass. IL-6 has been linked to β-cell dedifferentiation.42) Further, IL-6 KO mice fed a high-fat diet do not show an expansion of α-cell mass.42) Ipragliflozin reduced plasma IL-6 levels in rodent models of diabetes,38,40) which might underlie its role in the expansion of α-cells in obese T2DM db/db mice. Recently, β-cell dedifferentiation was described as a mechanism of β-cell failure and a cause of the imbalance in the ratio of α-cell to β-cell mass in humans43) and animal models.44,45) Ishida et al. reported that food restriction by pair feeding results in a reduction in β-cell dedifferentiation, as assessed by Foxo1 and Aldh1a3 immunohistochemistry in obese T2DM db/db mice.46) In addition, the food restriction increased the percentage of insulin-positive cells but not α-cells per islet.46) It is well-known that treatment with SGLT2 inhibitors results in glucosuria-related calorie loss, similar to food restriction. Therefore, calorie loss associated with ipragliflozin treatment might be correlated with the decreased ratio of α-cells to β-cells through a reduction in β-cell dedifferentiation. Further studies are needed to determine whether ipragliflozin affects pancreatic β-cell dedifferentiation in T2DM.

In conclusion, we demonstrated that treatment with ipragliflozin protects pancreatic islet β-cells from glucolipotoxicity and improves the imbalance in pancreatic α-cell and β-cell mass. Treatment with ipragliflozin may protect against the progressive loss of islet β-cells and maintain the proper balance of α-cell to β-cell ratio in patients with T2DM.

Acknowledgment The authors thank Drs. Atsuo Tahara and Masanori Yokono (Astellas Pharma Inc.), and Drs. Hiroshi Tomiyama, Akira Tomiyama, Yoshikiko Haino, and Yoshinori Kondo (Kotobuki Pharmaceutical Co., Ltd.) for their valuable comments and continuing encouragement.

Conflict of Interest The authors are employees of Astellas Pharma Inc.

REFERENCES

1) Abdul-Ghani MA, Tripathy D, DeFronzo RA. Contributions of beta-cell dysfunction and insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting glucose. *Diabetes Care*, 29, 1130–1139 (2006).
2) DeFronzo RA. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia*, 53, 1270–1287 (2010).
3) Lencioni C, Lupi R, Del Prato S. Beta-cell failure in type 2 diabetes mellitus. *Current Diabetes Reports*, 8, 179–184 (2008).
4) Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma A. Beta-cell adaptation and decapsulation during the progression of diabetes. *Diabetes*, 50 (Suppl. 1), S154–S159 (2001).
5) Kaneto H, Matsuoaka TA, Kimura T, Ohata A, Shimoda M, Kamei S, Mune T, Kaku K. Appropriate therapy for type 2 diabetes mellitus in view of pancreatic beta-cell glucose toxicity: “the earlier, the better.” *J. Diabetes*, 8, 183–189 (2016).
6) Iihara Y, Toyokuni S, Uchida K, Odaka H, Tanaka T, Ikeda H, Hiai...
39) Maedler K, Sergeev P, Ehres JA, Mathe Z, Bosco D, Berney T, Dayer JM, Reinecke M, Halban PA, Donath MY. Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 8138–8143 (2004).

40) Tahara A, Kurosaki E, Yokono M, Yamajaku D, Kihara R, Haya-shizaki Y, Takasu T, Imamura M, Li Q, Tomiyama H, Kobayashi Y, Noda A, Sasamata M, Shibasaki M. Effects of sodium-glucose cotransporter 2 selective inhibitor ipragliflozin on hyperglycaemia, oxidative stress, inflammation and liver injury in streptozotocin-induced type 1 diabetic rats. *J. Pharm. Pharmacol.*, **66**, 975–987 (2014).

41) Godoy-Matos AF. The role of glucagon on type 2 diabetes at a glance. *Diabetology & Metabolic Syndrome*, **6**, 91 (2014).

42) Ellingsgaard H, Ehres JA, Hammar EB, Van Lommel L, Quintens R, Martens G, Kerr-Conte J, Pattou F, Berney T, Pipeleers D, Halban PA, Schuit FC, Donath MY. Interleukin-6 regulates pancreatic alpha-cell mass expansion. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 13163–13168 (2008).

43) Cinti F, Bouchi R, Kim-Muller JY, Ohmura Y, Sandoval PR, Marsi M, Marselli L, Suleiman M, Ratner LE, Marchetti P, Accili D. Evidence of beta-cell dedifferentiation in human type 2 diabetes. *J. Clin. Endocrinol. Metab.*, **101**, 1044–1054 (2016).

44) Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. *Cell*, **150**, 1223–1234 (2012).

45) Wang Z, York NW, Nichols CG, Remedi MS. Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab.*, **19**, 872–882 (2014).

46) Ishida E, Kim-Muller JY, Accili D. Pair feeding, but not insulin, phloridzin, or rosiglitazone treatment, curtails markers of beta-cell dedifferentiation in db/db mice. *Diabetes*, **66**, 2092–2101 (2017).