Analysis of the Effect of Canagliflozin on Renal Glucose Reabsorption and Progression of Hyperglycemia in Zucker Diabetic Fatty Rats

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ABSTRACT

Sodium–glucose cotransporter 2 (SGLT2) plays a major role in renal glucose reabsorption. To analyze the potential of insulin-independent blood glucose control, the effects of the novel SGLT2 inhibitor canagliflozin on renal glucose reabsorption and the progression of hyperglycemia were analyzed in Zucker diabetic fatty (ZDF) rats. The transporter activity of recombinant human and rat SGLT2 was inhibited by canagliflozin, with 150- to 12,000-fold selectivity over other glucose transporters. Moreover, in vivo treatment with canagliflozin induced glycosuria in mice, rats, and dogs in a dose-dependent manner. It inhibited apparent glucose reabsorption by 55% in normoglycemic rats and by 94% in hyperglycemic rats. The inhibition of glucose reabsorption markedly reduced hyperglycemia in ZDF rats but did not induce hypoglycemia in normoglycemic animals. The change in urinary glucose excretion should not be used as a marker to predict the glycemic effects of this SGLT2 inhibitor. In ZDF rats, plasma glucose and HbA1c levels progressively increased with age, and pancreatic β-cell failure developed at 13 weeks of age. Treatment with canagliflozin for 8 weeks from the prediabetic stage suppressed the progression of hyperglycemia, prevented the decrease in plasma insulin levels, increased pancreatic insulin contents, and minimized the deterioration of islet structure. These results indicate that selective inhibition of SGLT2 with canagliflozin controls the progression of hyperglycemia by inhibiting renal glucose reabsorption in ZDF rats. In addition, the preservation of β-cell function suggests that canagliflozin treatment reduces glucose toxicity via an insulin-independent mechanism.

Introduction

Type 2 diabetes mellitus is characterized by hyperglycemia and relative insulin deficiency as a result of impaired insulin secretion from pancreatic β cells or insulin resistance. Chronic hyperglycemia leads to progressive impairment of insulin secretion, exacerbates insulin resistance, and worsens diabetes mellitus (Rossetti et al., 1990; Yki-Järvinen, 1992). Although various therapeutic drugs are available, it is still difficult to maintain long-term glycemic control in patients with type 2 diabetes (Kahn et al., 2006). It has been considered that progressive β-cell failure with exacerbation of diabetes mellitus attenuates the effects of conventional therapeutic drugs because the mechanism of these drugs is highly dependent on insulin action, either by stimulation of insulin secretion or by enhancement of insulin sensitivity. Therefore, continuous control of plasma glucose levels has been desired via mechanisms that are independent of insulin. Filtered glucose is reabsorbed via sodium–glucose cotransporters (SGLTs) in the luminal side of kidney tubules (Abdul-Ghani et al., 2011). SGLT2 is a low-affinity and high-capacity transporter located in the early S1 segment of the proximal tubule (Kanai et al., 1994); it is responsible for reabsorption of approximately 80–90% of glucose into blood. By contrast, SGLT1 in the late S3 segment of the proximal renal tubule achieves almost complete reabsorption of glucose from glomerular filtrates with a high affinity. Hence, SGLT2 and SGLT1 act in concert in the kidneys, and glucose is almost completely reabsorbed from the renal tubules and is barely detectable in urine of healthy adults (Abdul-Ghani et al., 2011).
Although mutations in the SGLT2 gene cause renal glycosuria (Santer et al., 2003), urinary glucose excretion (UGE) in subjects with dysfunctional SGLT1 is generally mild (Wright et al., 2007; Gorboulev et al., 2012), indicating that SGLT2 plays a critical role in renal glucose reabsorption. However, glucose reabsorption is only inhibited to 50% in subjects with normoglycemia or mild hyperglycemia, despite complete inhibition of SGLT2 (Komoroski et al., 2009; Abdul-Ghani et al., 2013). SGLT2 inhibitors efficiently lower plasma glucose levels independently of insulin action and secretion under hyperglycemic conditions (Oku et al., 1999; Liang et al., 2012; Ueta et al., 2014), whereas glucose reabsorption rates have not been investigated under hyperglycemic conditions. In addition, the effects of SGLT inhibition on the progression of metabolic abnormalities from the prediabetic normoglycemic stage have not been analyzed.

In this report, we characterized the pharmacologic properties of the novel SGLT2 inhibitor canagliflozin [TA-7284/JNJ-28431754; (1S)-1,5-anhydro-1-C-3(3L)-{(4-fluorophenyl)thiophen-2-yl)methyl}-4-methylphenyl]-6-glucitol hemihydrate] and examined the effects of SGLT2 inhibition on renal glucose reabsorption in normoglycemic and hyperglycemic animals. Subsequently, we investigated the effects of insulin-independent control of plasma glucose levels on the progression of hyperglycemia in Zucker diabetic fatty (ZDF) rats, which develop metabolic abnormalities similar to those in patients with type 2 diabetes.

Materials and Methods

Reagents and Chemicals

Canagliflozin was synthesized at the Medicinal Chemistry Laboratory at Mitsubishi Tanabe Pharma Corporation (Toda-shi, Saitama, Japan) (Nomura et al., 2010). All other chemicals were of reagent or tissue-culture grade.

Cell-Based Assays

Sodium-Dependent Monosaccharide Uptake in Chinese Hamster Ovary Cells Expressing SGLT1, SGLT2, SGLT4, SGLT6, and Sodium/Myo-Inositol Transporter SMIT1. Expression plasmids containing human SGLTs (hSGLT1, hSGLT2, hSGLT4, hSGLT6, and sodium/myo-inositol transporter hSMIT1), rat SGLTs (rSGLT1 and rSGLT2), and mouse SGLTs (mSGLT1 and mSGLT2) were stably transfected into Chinese hamster ovary (CHO-K1) cells. Cells were seeded into Opti-96 plates (PerkinElmer, Waltham, MA) and were incubated at 37°C in assay buffer containing 50 mM HEPES, 20 mM Tris Base, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 137 mM NaCl at pH 7.4. SGLT1, SGLT2, and SGLT4 transport activity was assayed after 1- to 3-hour treatments with 300–500 μM α-methyl-D-glucopyranoside (AMG; Sigma-Aldrich, St. Louis, MO) in the presence of [14C]AMG (PerkinElmer or Amersham). Assays of myo-inositol (MI) transport activity of hSGLT6 and hSMIT1 were performed after labeling cells with [3H]MI (PerkinElmer) for 2 hours in the presence of 30 μM MI (Sigma-Aldrich) and 800 μM MI, respectively. Radioactive counts were assessed in cells using a TopCount instrument (Packard Instruments, Meriden, CT).

Glucose Transporter GLUT1, GLUT2, GLUT4, and GLUT5 Activities. Glucose transporter GLUT1 activity was assayed in L6 cells (Health Science Research Resources Bank, Osaka, Japan) cultivated in Krebs-Ringer-Phosphate-HEPES buffer containing 150 mM NaCl, 5 mM KCl, 1.25 mM MgSO₄, 1.25 mM CaCl₂, 10 mM HEPES, and 2.9 mM Na₃HPO₄ at pH 7.4. Cells were treated with 750 μM 2-deoxyglucose (2-DG) and [14C]2-DG for 15 minutes at room temperature, and incorporated radioactivity was determined using a liquid scintillation counter (Packard Instruments, Boston, MA). Protein contents were determined using the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL).

GLUT2 activity was determined in HepG2 hepatoma cells (American Type Culture Collection, Manassas, VA) in Krebs buffer containing 0.2% bovine serum albumin. Cells were treated with 1 mM glucose and 2.5 μCi [3H]-2-DG for 15 minutes at 37°C. Glutamate-mediated glucose uptake was determined in primary human adipocytes (ZenBio, Durham, NC) in assay buffer containing 0.2% bovine serum albumin. Cells were treated with 10 nM insulin, 1 mM glucose, and [14C]2-DG (PerkinElmer) for 30–40 minutes at 37°C. CHO cells expressing human GLUT5 were treated with 3 mM fructose (Sigma-Aldrich) and 0-[U-14C]fructose (ARC, St. Louis, MO) for 20 minutes at 37°C. Radioactive counts in the cells were assessed using a TopCount instrument (Packard Instruments).

Binding to Various Receptors, Channels, and Transporters.

In vitro radioligand binding assays of canagliflozin activities were performed for a panel of 50 rat, guinea pig, and human receptors (Cerep, Cell l’Evescault, France). Canagliflozin was tested at 1 and 10 μM. Assay details are presented in Supplemental Table 1.

In Vivo Studies

Animals and Canagliflozin Administration. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation or the Janssen Research Development Institutional Animal Care and Use Committee.

Experimental animals were housed with a 12-hour light/dark cycle and controlled temperature and humidity and were provided water ad libitum and a standard commercial diet. Canagliflozin was prepared in 0.5% hydroxypropyl methylcellulose for oral gavage at 10 ml/kg. For long-term treatment studies, canagliflozin was mixed with standard chow (CRF1; Oriental Yeast, Tokyo, Japan).

UGE in C57BL/6J Mice, Sprague-Dawley Rats, Beagle Dogs, and 5/6 Nephrectomized Rats under Fed Conditions. Male 6- to 7-week-old C57BL/6J mice (n = 4; CLEA Japan, Tokyo, Japan) were treated with canagliflozin at 0.3–100 mg/kg p.o. and were given free access to water and chow. Using metabolic cages, 24-hour urine samples were collected. Male 6-week-old Sprague-Dawley rats (n = 8; Japan SLC, Shizuoka, Japan) were treated with canagliflozin at 0.3, 1, 3, 10, and 30 mg/kg p.o., and urinary glucose contents were determined in 24-hour urine collected in metabolic cages. Concomitantly, 350 μl of venous blood was collected from jugular veins of conscious satellite animals (n = 4) at 0.5, 2, 4, 6, 8, and 24 hours after administration. Canagliflozin (0.3, 1, and 3 mg/kg) was administered p.o. immediately before food was given in the morning to 7- to 8-month-old male beagle dogs (Kitayama Labes, Nagano, Japan), and urine was collected for 24 hours. Venous blood (0.5 ml) was collected at 0, 0.5, 2, 4, 6, 8, and 24 hours after administration.

In separate experiments, 5-week-old male Sprague-Dawley rats were 5/6-nephrectomized (Nx) or sham operated 3 weeks prior to the experiment at Japan SLC and were treated with 0.3, 3, or 30 mg/kg canagliflozin p.o. Animals were then kept in metabolic cages with free access to water and chow, and urine was collected for over 24 hours.

Renal Glucose Absorption in Normoglycemic Sprague-Dawley Rats. Canagliflozin (0, 1, 3, 10, and 30 mg/kg) was orally administered to 6-week-old male Sprague-Dawley rats, which were kept in metabolic cages with free access to water and chow. Urine was collected over 24 hours, and 1-ml samples of venous blood were collected from jugular veins at 24 hours after administration. Concentrations of glucose and creatinine were determined in urine and plasma samples, and urine volumes were measured to calculate the transport of glucose (TG). Additional experiments were performed to calculate inhibition rates of reabsorption. TG (in grams per kilogram per hour) was defined as the difference between the renal glucose filtration rate (RGF = Plasma glucose level × Creatinine clearance) and UGE. Maximal transport of glucose (TmG) in the presence of the inhibitor is presented as TmG(+).
Renal Glucose Reabsorption and Plasma Glucose Levels in Diabetic ZDF Rats. Canagliflozin (0, 0.3, 3, and 30 mg/kg) was orally administered to 9- to 10-week-old male ZDF rats under fed conditions (n = 8 to 9; ZDF-Lepr<sup>fa/CrlCrj</sup> [Lepr<sup>fa</sup>/Lepr<sup>fa</sup>]; Charles River Laboratories Japan, Yokohama, Japan.) Urine samples were collected at 0–1, 1–2, 2–4, and 4–6 hours after dosing, and plasma glucose levels were determined. At 6 hours after dosing, 1-ml aliquots of venous blood were collected from jugular veins, and plasma creatinine concentrations were determined. The concentrations of glucose and creatinine in urine and plasma samples were determined, and urine volumes were measured to calculate TG and reabsorption inhibition rates.

Effects on Plasma Glucose Levels in Diabetic ZDF and Normoglycemic ZCL Rats. Canagliflozin (0, 1, 3, or 10 mg/kg) was orally administered to 13-week-old male ZDF rats (n = 6) and their lean littermates (ZCL rats) (n = 6; ZDF-Lepr<sup>fa/CrlCrj</sup> [?/?]; Charles River Laboratories Japan) rats under fed conditions. Blood samples were collected from tail veins at 0, 1, 2, 4, 6, and 24 hours, and concentrations of glucose in plasma were determined. In addition, 150-μl aliquots of venous blood were collected from tips of tails at 2, 6, and 24 hours after dosing, and concentrations of canagliflozin in plasma were determined.

Long-Term Treatments in 5-Week-Old ZDF Rats. Five-week-old male ZDF rats (n = 8) were fed a canagliflozin mixture diet [0, 0.003, 0.01, and 0.03% (w/w)] for 8 weeks. Age-matched ZCL rats (n = 6) were used as controls. Blood samples of 70 μl were collected from tail veins once a week up to 13 weeks of age, and plasma concentrations of glucose, plasma concentrations of insulin, and HbA1c levels were determined. At 13 weeks of age, animals were anesthetized using 20 mg/animal of pentobarbital sodium, blood was collected from the abdominal vena cava, and pancreatic tissues were excised. Portions of pancreatic tissues were weighed and stored at −80°C prior to determination of insulin contents. Separate portions of the pancreatic tissue were fixed in 10% neutralized buffered formalin and were processed for histologic and immunohistochemical examinations.

Determination of Glucose, Insulin, HbA1c, and Creatinine Levels. Concentrations of glucose in plasma were measured using the Accurus Auto CRE 7170 Kit (Shima-Test Corporation, Tokyo, Japan). Concentrations of glucose in urine were measured using the Accuras Auto CRE 7170 Kit (Shima-Test Corporation, Tokyo, Japan). Concentrations of glucose in urine were measured using the Glucose C-II test (Wako Pure Chemical Industries, Osaka, Japan). Concentrations of insulin in plasma were measured using the Tosoh Automated Glycoheamoglobin Analyzer (Tosoh, Tokyo, Japan). Concentrations of creatinine in plasma were measured using the SEROTEC UGLU-L Kit (Serotec Co., Ltd., Sapporo, Japan). Concentrations of creatinine in urine were measured using the Accuras Auto CRE 7170 Kit (Shino-Test Corporation, Tokyo, Japan). Concentrations of glucose in plasma were measured using the Glucose C-II test (Wako Pure Chemical Industries, Osaka, Japan). Concentrations of glucose in urine were measured using the SEROTEC UGLU-L Kit (Serotec Co., Ltd., Sapporo, Japan).

Pharmacokinetics. Plasma canagliflozin was extracted using OASIS HLB µElution 96-well plates (30 μm; Waters Corporation, Milford, MA). After elution with acetonitrile, canagliflozin contents were determined using a validated liquid chromatography–tandem mass spectrometry method with an API4000 mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with a Cadenza CD C-18 column (3 μm, 2.0 × 50 mm; Imtakt Co., Ltd., Kyoto, Japan).

Histology and Immunohistochemistry. Fixed tissues were embedded in paraffin for histologic examinations. Sections of pancreatic tissues were stained with H&E. For immunohistochemical analyses, sections were treated with anti-insulin guinea pig polyclonal primary antibody (Dako, Glostrup, Denmark) followed by horseradish peroxidase–conjugated secondary antibody (anti-guinea pig IgG goat polyclonal antibody; Millipore, Billerica, MA) and were stained with 3,3′-diaminobenzidine (Kakimoto et al., 2013). Stained sections were then examined under a light microscope (Olympus BX51; Olympus, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± S.E.M. or S.D., or with 95% confidence intervals. IC<sub>50</sub> values were calculated using regression analyses. Statistical analyses were performed using an SAS-based system (SAS Institute, Cary, NC) or Prism software (GraphPad, San Diego, CA), and significant differences were identified using parametric Dunnett’s multiple comparisons.

Results

SGLT2 and SGLT1 Inhibition. Canagliflozin inhibited AMG uptake with IC<sub>50</sub> values of 4.2 ± 1.5, 3.7 ± 0.2, and 5.6 ± 1.1 nM in human, rat, and mouse SGLT2-expressing cells, respectively (Table 1). Lineeweaver–Burk plots revealed competitive inhibition with canagliflozin, with a K<sub>i</sub> value of 0.84 ± 0.06 nM for hSGLT2. AMG uptake was inhibited by canagliflozin, with IC<sub>50</sub> values of 663 ± 180 nM, 555 ± 31 nM, and 613 ± 56 nM in human, rat, and mouse SGLT1-expressing cells, respectively. These data indicate that canagliflozin inhibits SGLT2s with 109- to 158-fold selectivity over SGLT1s (Table 1).

Selectivity over Other SGLTs and GLUTs. Na<sup>+</sup>-dependent AMG uptake was not inhibited by canagliflozin in CHO cells stably expressing hSGLT4. Although canagliflozin inhibited Na<sup>+</sup>-dependent MI uptake in hSGLT6-expressing cells with an IC<sub>50</sub> of approximately 3 μM, hSMIT1 activity was not affected (Table 2).

Canagliflozin did not inhibit uptake of 2-DG in L6 myoblast cells and HepG2 cells at concentrations up to 10 and 50 μM, respectively. In primary human adipocytes, canagliflozin inhibited insulin-stimulated 2-DG uptake with an IC<sub>50</sub> of approximately 7 μM; however, it did not inhibit fructose uptake at concentrations of up to 20 μM in CHO cells stably expressing GLUT5 (Table 3). These data indicate that canagliflozin inhibits human, rat, and mouse SGLT2s with >100-fold selectivity over SGLT1 and with >1000-fold selectivity over other Na<sup>+</sup>-dependent and facilitative GLUTs.

Receptor Binding Screen. Effects of canagliflozin on ligand binding to a panel of 50 rat, guinea pig, or human receptors were evaluated at 1 and 10 μM (Supplemental Table 1). Canagliflozin inhibited ligand binding to the human A<sub>1</sub> receptor (62%), the human norepinephrine transporter (51%), and the human 5HT<sub>2A</sub> receptor (56%) at 10 μM; however, binding inhibition was ≤30% for most other receptors (Supplemental Table 1). These data indicate >1000-fold selectivity of canagliflozin for SGLT2.

Effects on UGE in Normal Mice and Dogs. Although glucosuria was not detected in vehicle-treated C57BL/6J mice, canagliflozin dose-dependently increased UGE over 24 hours with significant effects at ≥10 mg/kg (Fig. 1A). In vehicle-treated Sprague-Dawley rats, UGE was not detected in urine collected over 24 hours. By contrast, canagliflozin

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<th>IC&lt;sub&gt;50&lt;/sub&gt; Ratio of SGLT1/SGLT2</th>
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<th>nM</th>
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<tbody>
<tr>
<td>Human</td>
<td></td>
<td>4</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td>3</td>
<td>3.7 ± 0.2</td>
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<tr>
<td>Mouse</td>
<td></td>
<td>3</td>
<td>5.6 ± 1.1</td>
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increased UGE in a dose-dependent manner, with statistically significant effects at ≥1 mg/kg. In satellite animals, plasma concentrations of canagliflozin increased with dose increments (Fig. 1, B and C).

In dogs, canagliflozin dose-dependently increased UGE at 0.3, 1, and 3 mg/kg, with significant effects at doses of ≥0.3 mg/kg (P < 0.01). Plasma concentrations of canagliflozin and UGE were highly correlated after doses of 0.3, 1, and 3 mg/kg (Fig. 1D). Pearson’s correlation coefficients (with 95% confidence intervals) between the pharmacokinetic parameters and UGE were 0.951 (0.832–0.987), 0.962 (0.867–0.990), and 0.956 (0.847–0.988) for Cmax, area under the concentration-time curve from time 0 to 24 hours (AUC0–24 h), and from time 0 to infinity (AUC0–∞), respectively.

Canagliflozin induced glucosuria in a dose-dependent manner in Nx and sham-operated rats, and creatinine clearance was decreased to approximately 50% after nephrectomy. Moreover, canagliflozin-mediated increases in UGE levels were lower in Nx rats than in sham rats. However, no differences in glucosuria per urinary creatinine levels were observed between Nx and sham-operated rats (Fig. 2A).

These results indicate that canagliflozin increases UGE levels in several species in a dose-dependent and plasma concentration-dependent manner and that the induction of glucosuria is dependent on glomerular filtration rates.

Effects on Renal Glucose Reabsorption and UGE in Normal Rats. In initial experiments, no dose-related effects on RGF were observed after treatments with canagliflozin. However, TG in the presence of inhibitor (TG[+]i) was dose-dependently decreased and was 0.330 g/kg per hour at the highest dose (Fig. 2B). Accordingly, compared with the vehicle group, reabsorption of glucose was significantly reduced by canagliflozin at doses of ≥1 mg/kg, and TG[+]i was equal to TmG[i] in canagliflozin-treated animals with detectable UGE. Although inhibition rates of glucose reabsorption (1 – TG[+]i/TG[−]) increased in a dose-dependent manner (where TG[−] is TG in the absence of inhibitor), the maximum inhibition was approximately 50–55% at doses of 10 and 30 mg/kg (Fig. 2C).

Table 2
Inhibitory effect of canagliflozin on human SGLT3, SGLT4, SGLT6, and SMIT1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
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<tr>
<td>Canagliflozin</td>
<td>&gt;10</td>
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*Data from Liang et al., 2012.

![Fig. 1. UGE in normoglycemic mice, rats, and dogs. (A) Male C57BL/6J mice in the fed state; 24-hour urine samples were collected in metabolic cages. Values are expressed as the mean ± S.E.M. (n = 4). (B) Urine samples were collected from 6-week-old Sprague-Dawley rats in metabolic cages for 24 hours. Data are expressed as the mean ± S.E.M. (n = 4). (C) Blood was collected from tail veins of 6-week-old Sprague-Dawley rats at 0.5, 2, 4, 6, 8, and 24 hours after oral administration of canagliflozin. Data are expressed as the mean ± S.D. (n = 4). (D) Canagliflozin was administered to dogs, and blood was collected at 0.5, 2, 4, 6, 8, and 24 hours, and urine was collected over 24 hours. UGE levels were plotted against AUC0–24 h of concentrations of canagliflozin in plasma. Differences from vehicle-treated controls were identified using Dunnett’s multiple comparison test (**P < 0.01). AUC0–24 h area under the concentration-time curve from time 0 to 24 hours.

Effects on Renal Glucose Reabsorption and Plasma Glucose Levels in ZDF Rats. Plasma glucose levels in 9- to 10-week-old ZDF rats were 527.3 ± 11.2 mg/dl before treatment with canagliflozin or vehicle. In vehicle-treated ZDF rats, UGE was 0.612 g/kg per hour over 6 hours (Fig. 3). Single oral treatment with canagliflozin decreased plasma glucose levels in a dose-dependent manner, and this was significant at doses of 0.3 mg/kg (Fig. 3A). UGE was increased by canagliflozin treatment; however, this effect was only significant at a dose of 30 mg/kg (Fig. 3B). Nonetheless, TG[+]i was dose-dependently decreased to 0.302 g/kg per hour at the maximum dose (Fig. 3C). Plasma glucose levels were decreased, and RGF was accordingly reduced after canagliflozin treatment. Analysis of temporal changes revealed that inhibition rates of glucose reabsorption were significantly increased at 2 hours after treatment with 3 and 30 mg/kg, and the maximum inhibition rate was approximately 95% (Fig. 3D).

Doses of 1, 3, and 10 mg/kg canagliflozin markedly reduced plasma glucose levels in ZDF rats (Fig. 4A) but only slightly reduced those in normoglycemic ZCL rats (Fig. 4B). Unbound plasma canagliflozin was similarly increased in a dose-dependent manner, and after treatment with doses of 10 mg/kg, the maximum concentrations were 118.3 and 95.0 nM in ZDF and ZCL, respectively. These concentrations of unbound canagliflozin were far below the IC50 value for rSGLT1.
However, the plasma unbound concentrations of canagliflozin were 2.9 and 4.5 nM at 24 hours after 3 mg/kg treatments in ZDF and ZCL rats, respectively, and were similar to the IC_{50} value for rSGLT2 (Fig. 4C). These data indicate that canagliflozin strongly inhibits the SGLT2-mediated reabsorption of glucose and reduces plasma glucose levels in hyperglycemic ZDF rats.

**Effects of 8-Week Canagliflozin Treatments on HbA1c Levels and Pancreatic β-Cell Exhaustion in 5-Week-Old ZDF Rats.** Before canagliflozin treatment, plasma glucose and insulin levels in 5-week-old ZDF rats were 120.2 mg/dl and 5.21 ng/ml, respectively. In control ZDF rats, plasma glucose levels gradually increased at 6 to 7 weeks of age, rapidly increased from 8 weeks, and reached a steady level of approximately 600 mg/dl at 11–13 weeks (Fig. 5, A and B). HbA1c levels were also markedly increased at 13 weeks of age (Fig. 5C). Plasma insulin levels were transiently elevated at 6 to 7 weeks of age and then gradually decreased after 8 weeks. In ZCL rats, plasma glucose levels were 98.3 and 151.5 mg/dl at 5 and 13 weeks of age, respectively, and HbA1c levels remained almost unchanged during this period. Plasma insulin levels were gradually increased by 2.94 ng/ml from 5 to 13 weeks of age.

Dietary canagliflozin at 0.003, 0.01, and 0.03% (w/w) is equivalent to doses of 3.1, 10.0, 28.4 mg/kg per day p.o., respectively. Increases in plasma glucose and HbA1c levels were suppressed for 8 weeks in 5-week-old canagliflozin-treated ZDF rats, and plasma glucose and HbA1c levels were similar to those of 13-week-old ZCL rats. Temporal increases in plasma insulin levels at 6–8 weeks were significantly suppressed, and plasma insulin levels gradually increased (Fig. 5B). This increase in plasma insulin levels was greater in lower dose groups, and plasma concentrations of insulin were higher in all canagliflozin-treated rats than in control ZDF rats from 10 weeks of age. Although food intake did not differ between canagliflozin-treated and control ZDF rats throughout the study (Supplemental Fig. 1A), the body weight was lower in the canagliflozin-treated group than in the control group up to 10 weeks of age. Thereafter, canagliflozin-treated rats, but not control ZDF rats, gained weight (Supplemental Fig. 1B). HbA1c levels in canagliflozin-treated ZDF rats were significantly lower than those in control ZDF rats (Fig. 5C).

At 13 weeks of age, pancreatic insulin contents in control ZDF rats were significantly lower than those in ZCL rats. Moreover, insulin contents in canagliflozin-treated ZDF rats were significantly higher than those in control ZDF rats and were similar to those in ZCL rats (Fig. 6A).

Immunohistochemical assessments of pancreatic tissues revealed that although insulin immunoreactivity was strong in pancreatic islets from ZCL rats, it was markedly decreased in ZDF rats (Fig. 6, Ba and Bb). In canagliflozin-treated ZDF rats, insulin immunoreactivity in pancreatic islets was similar to that in ZCL rats (Fig. 6C).

In histologic assessments, the islets of ZDF rats showed β-cell vacuolation that was indicative of degenerative changes (Fig. 7, A and B). By contrast, vacuolation of β cells was not present in the islets of ZCL rats. Hence, orally administered canagliflozin reduced the incidence of β-cell vacuolation (Fig. 7, C–E).
Canagliflozin selectively inhibited the transporter activity of human, rat, and mouse SGLT2. At 24 hours after oral administration of 3 mg/kg canagliflozin, unbound plasma concentrations were similar to the IC50 value for SGLT2, and the Cmax value was far lower than the IC50 value for SGLT1 after treatment with 30 mg/kg canagliflozin. Although temporal inhibition of intestinal SGLT1 may follow direct exposure to the present drug formulation, renal SGLT2 activity was selectively inhibited for 24 hours after oral administration of canagliflozin in rats. In accordance with the inhibition of SGLT2, UGE was dose-dependently increased in mice, rats, and dogs. Moreover, pharmacokinetic and pharmacodynamic analyses revealed plasma concentration-dependent effects on UGE. Similar ratios of UGE per creatinine clearance between normal and Nx rats indicated that UGE is dependent on glomerular filtration rate. These data demonstrate the utility of canagliflozin for analyses of the role of SGLT2 inhibition in glucose reabsorption and progression of hyperglycemia in vivo.

Although the maximum inhibition rates of glucose reabsorption differed between Sprague-Dawley and ZDF rats (Figs. 2C and 3D), TG(+) was approximately 0.3 g/kg per hour in both animal groups (Figs. 2B and 3C). Given the extensive inhibition of SGLT2, the remaining TG (TG(-)) likely reflects the activity of SGLT1 (Abdul-Ghani et al., 2013; Rieg et al., 2014).

Accordingly, reabsorption is sustained at the level of SGLT1-mediated TG. It remains paradoxical that although 80–90% of reabsorption is mediated by SGLT2, SGLT2 inhibitors reduce filtered glucose loads by only 30–50% in humans (Abdul-Ghani et al., 2013). Similarly, glucose excretion in SGLT2-knockout mice comprises approximately 60% of filtrated glucose (Gorboulev et al., 2012), and only 16–50% inhibition of glucose reabsorption has been achieved in clinical studies of SGLT2 inhibitors (Koroski et al., 2009; Abdul-Ghani et al., 2013). In all of these studies, the maximum inhibition of reabsorption of only approximately 50% was demonstrated under normoglycemic and mild hyperglycemic conditions. Thus, the inhibition of SGLT2 may lead to full utilization of SGLT1, which is located in more distal segments of proximal tubules (Abdul-Ghani et al., 2011). In this study, reabsorption rates, which are calculated as TG(+) / TG(-), were dose-dependently decreased to approximately 45% at the maximum dose in Sprague-Dawley rats. In normoglycemic rats, inhibition of SGLT2 resulted in up to 55% inhibition of glucose reabsorption at the maximum dose (Fig. 8A). By contrast, at plasma glucose levels of >200 mg/dl, TG(-) levels were equal to those of TmG. Moreover, the TG(+) / TmG ratio was less than 20%, indicating greater than 80% inhibition in ZDF rats. Thus, extrapolation of these data suggests higher inhibition rates of glucose reabsorption in hyperglycemic human subjects than in normoglycemic ones (Fig. 8B).

UGE increased after canagliflozin treatment in both normoglycemic and hyperglycemic rats. In normoglycemic animals, canagliflozin significantly increased UGE, whereas plasma glucose levels were minimally affected. Although plasma glucose levels were dose-dependently reduced in hyperglycemic animals, UGE was only affected at 6 hours after treatment with the highest dose. The dissociation between increased UGE and reduced plasma glucose levels in
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Therapeutic SGLT2 inhibition for type 2 diabetes patients may produce favorably low risks of hypoglycemia, as indicated by high glucose-lowering activity in hyperglycemia and low glucose-lowering activity in normoglycemia. SGLT2 activity is similarly inhibited in ZDF, Sprague-Dawley, and ZCL rats at doses that correspond to concentrations of unbound canagliflozin in plasma. Inhibition of TmG was greater in hyperglycemic animals than in normoglycemic animals. Previous studies (Abdul-Ghani et al., 2013) in normoglycemic rats and humans have demonstrated inhibition of reabsorption based on TmG[+] and TmG[−] levels. Because TmG[−] levels are dependent on PG, they remain less than and equal to TmG under normoglycemic and hyperglycemic conditions, respectively. In ZDF animals, the ratio of TmG[+/−] was highly correlated with the reduction of plasma glucose levels. By contrast, TmG[+/+] = TmG[−] was as high as 45% and TmG[−] was only moderately (0.5 g/kg per hour) inhibited in normoglycemic rats. As described above, the hypoglycemic effects of moderate inhibition of glucose reabsorption can be compensated by hepatic glucose production. Hence, the effects of moderate inhibition of glucose reabsorption may not be sufficient to produce hypoglycemia in normoglycemic individuals, whereas extensive inhibition of glucose reabsorption is correlated with antihyperglycemic effects under hyperglycemic conditions. These observations indicate that canagliflozin controls plasma glucose levels independently of insulin.

The effect of plasma glucose control using an insulin-independent mechanism on the progression of hyperglycemia was determined in diabetic model rats. ZDF rats develop progressive metabolic abnormalities similar to those in obese patients with type 2 diabetes. Accordingly, insulin resistance and plasma insulin levels were elevated at 7 weeks of age, and plasma insulin levels were subsequently decreased with the development of hyperglycemia at 10 weeks. At 13 weeks of age, vehicle-treated ZDF rats exhibited extensive hyperglycemia, which was accompanied by a decline in plasma insulin levels. Treatment with canagliflozin from the prediabetic stage (5 weeks old) suppressed the progression of hyperglycemia, indicating the potential of SGLT2 inhibitors to control....

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**Fig. 6.** Effects of 8-week treatments with canagliflozin on abnormalities of islet structure in ZDF rats. Canagliflozin [0, 0.003, 0.01, and 0.03% (w/w)] in diet was administered to ZDF rats (5 weeks old at start) for 8 weeks. (A) Pancreatic insulin contents in ZDF rats [0.003% (c), 0.01% (d), and 0.03% (e)] were immunostained for insulin. Bar, 100 μm.

**Fig. 7.** Effects of 8-week treatments with canagliflozin on abnormalities of islet structure in ZDF rats. Canagliflozin [0, 0.003, 0.01, and 0.03% (w/w)] in diet was administered to ZDF rats (5 weeks old at start) for 8 weeks (n = 6–8). Pancreatic islets from ZCL/control rats (A), ZDF/control rats (B), and ZDF/canagliflozin-treated rats [0.003% (C), 0.01% (D), and 0.03% (E)] were stained with hematoxylin and eosin. Arrows indicate β-cell vacuolation. Bar, 100 μm.

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normoglycemic rats may indicate a compensatory mechanism involving hepatic glucose production. Accordingly, endogenous glucose production is approximately 0.36–0.41 g/kg per hour in normal rats (Torres et al., 2009; Ueta et al., 2014). Canagliflozin treatments increased UGE in ZDF rats. In hyperglycemic animals, RGF is higher than TmG, and nontransported glucose appears as glucosuria in urine. The inhibition of SGLT2 reduces TmG and further increases nonreabsorbed glucose levels. By contrast, decreased reabsorption leads to decreased plasma glucose levels, and RGF decreases accordingly. Because both RGF and TmG are reduced after inhibition of SGLT2, changes in UGE do not accurately reflect inhibition of TG (Fig. 8B). Thus, although the inhibitory effect of canagliflozin on TmG was dose dependent, net UGE increases were not dose dependent under hyperglycemic conditions. Thus, the inhibitory effects of canagliflozin on glucose reabsorption may reflect plasma glucose levels as reported in a previous study (Liang et al., 2012), suggesting that changes in UGE fail to indicate glycemic effects of SGLT2 inhibitors.

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blood glucose levels in type 2 diabetes through mechanisms unrelated to insulin. In ZDF rats, plasma insulin levels were transiently increased at 7 weeks of age and subsequently decreased. Moreover, extensive reduction of pancreatic insulin contents in vehicle-treated ZDF rats suggested the development of pancreatic exhaustion. By contrast, plasma insulin levels gradually increased over time in canagliflozin-treated ZDF rats, although this increase was suppressed in a dose-dependent manner. Transiently elevated insulin levels at 7 weeks of age may reflect mechanisms that compensate for extensive insulin resistance. Thus, treatment of canagliflozin likely reduces insulin requirements and maintains blood glucose levels in the normal range.

Histopathologic analysis revealed structural changes in pancreatic islets in ZDF rats. Accordingly, long-term hyperglycemia induces structural disorganization of pancreatic islets (Jones et al., 2010). After the present 8-week treatments with canagliflozin, pancreatic insulin contents were similar to those in normal rats. In a previous study, hyperglycemia suppressed β-cell function through glucotoxic mechanisms (Rossetti et al., 1990) and caused pancreatic exhaustion owing to chronic oversecretion of insulin in response to high blood glucose levels. Structural changes of pancreatic islets were also reduced in canagliflozin-treated ZDF rats, although this increase was suppressed in a dose-dependent manner. Transiently elevated insulin levels at 7 weeks of age may reflect mechanisms that compensate for extensive insulin resistance. Thus, treatment of canagliflozin likely reduces insulin requirements and maintains blood glucose levels in the normal range.

Fig. 8. Schematic representation of the effects of canagliflozin on glucose transport in normoglycemic and hyperglycemic rats. (A) In normoglycemic Sprague-Dawley rats, glucose in filtrates is mainly transported by SGLT2, and TG[−] levels are maintained below those of TmG (TG[−] < TmG). Canagliflozin reduces TG levels (red arrow) from TG[−] to TG[+], for which SGLT1 mediates transport. The inhibition rate of reabsorption (1 − TG[+] / TG[−]) was ≤55%. (B) In hyperglycemic ZDF rats, TG[−] levels were equal to TmG levels (TG[−] = TmG), reflecting RGFs that were above TmG. TG[−] is mediated by both SGLT1 and SGLT2. Complete inhibition of SGLT2 activity reduces TG level from TG[−] to TG[+], but it is sustained at the level of SGLT1-mediated TG. The inhibition rate of reabsorption (1 − TG[+] / TmG) was approximately 80%. SD, Sprague-Dawley.

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