Intestinal Sodium Glucose Cotransporter 1 Inhibition Enhances Glucagon-Like Peptide-1 Secretion in Normal and Diabetic Rodents

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ABSTRACT

The sodium glucose cotransporter (SGLT) 1 plays a major role in glucose absorption and incretin hormone release in the gastrointestinal tract; however, the impact of SGLT1 inhibition on plasma glucagon-like peptide-1 (GLP-1) levels in vivo is controversial. We analyzed the effects of SGLT1 inhibitors on GLP-1 secretion in normoglycemic and hyperglycemic rodents using phloridzin, CGMI [3-(4-cyclopropylphenylmethyl)-1-(β-D-glucopyranosyl)-4-methylindole], and canagliflozin. These compounds are SGLT2 inhibitors with moderate SGLT1 inhibitory activity, and their IC50 values against rat SGLT1 and mouse SGLT1 were 609 and 760 nM for phloridzin, 39.4 and 41.5 nM for CGMI, and 555 and 613 nM for canagliflozin, respectively. Oral administration of these inhibitors markedly enhanced and prolonged the glucose-induced plasma active GLP-1 (aGLP-1) increase in combination treatment with sitagliptin, a dipeptidyl peptidase-4 (DPP4) inhibitor, in normoglycemic mice and rats. CGMI, the most potent SGLT1 inhibitor among them, enhanced glucose-induced, but not fat-induced, plasma aGLP-1 increase at a lower dose compared with canagliflozin. Both CGMI and canagliflozin delayed intestinal glucose absorption after oral administration in normoglycemic rats. The combined treatment of canagliflozin and a DPP4 inhibitor increased plasma aGLP-1 levels and improved glucose tolerance compared with single treatment in both 8- and 13-week-old Zucker diabetic fatty rats. These results suggest that transient inhibition of intestinal SGLT1 promotes GLP-1 secretion by delaying glucose absorption and that concomitant inhibition of intestinal SGLT1 and DPP4 is a novel therapeutic option for glycemic control in type 2 diabetes mellitus.

Introduction

Long-term hyperglycemia leads to toxic effects in various tissues and organs (Rossetti et al., 1990; Leahy et al., 1992; Nathan, 1993), and therapeutic strategies to treat patients with type 2 diabetes mellitus (T2DM) are currently focused on controlling blood glucose levels (Blonde, 2010; Bennett et al., 2012; Inzucchi et al., 2012; Qaseem et al., 2012). Many classes of oral antihyperglycemic drugs (OADs) have been developed, but it is still difficult to maintain long-term glycemic control in most patients with T2DM because of the limited efficacy and safety issues of drugs (Kahn et al., 2006; Blonde, 2010; Qaseem et al., 2012). Recently, multiple functions of incretins, such as glucagon-like peptide-1 (GLP-1), have been investigated. GLP-1 is secreted from enteroendocrine L cells in the distal part of the small intestine in response to dietary stimulation (Orskov et al., 1993) and exerts antidiabetic effects, such as enhancing glucose-dependent insulin secretion and inhibiting gastric emptying, food intake, and glucagon secretion (Drucker, 2003). Dipeptidyl peptidase-4 (DPP4) inhibitors, a novel class of OADs, delay enzymatic degradation and inactivation of GLP-1, thereby increasing insulin release and decreasing glucagon secretion in a glucose-dependent manner (Drucker, 2003; Fukuda-Tsuku et al., 2012).

Sodium glucose cotransporter (SGLT) 2 is predominantly located on the apical side of renal proximal tubules and plays a critical role in glucose reabsorption from glomerular filtrates (Kanai et al., 1994a; Hediger et al., 1995; You et al., 1995; Abdul-Ghani et al., 2013). SGLT2 inhibitors, the newest class of OADs, lower plasma glucose levels independent of insulin action by inhibiting renal glucose reabsorption (Oku et al., 1999, 2000; Arakawa et al., 2001; Ueta et al., 2005). On the other hand, SGLT1 is highly expressed on the brush-border membrane of villus enterocytes in the proximal part of the small intestine and is responsible for dietary glucose absorption (Takata et al., 1992; Freeman et al., 1993; Kanai et al., 1994b). Glucose stimulates GLP-1 secretion from...
the gastrointestinal (GI) tract, and there is an increasing body of evidence that SGLT1 is a sensor linking glucose to incretin release (Moriya et al., 2009; Gorboulev et al., 2012). However, the observation that the SGLT1 inhibitor increased portal GLP-1 levels after intestinal glucose infusion contradicts this hypothesis (Shibazaki et al., 2012). The relative contributions of SGLT1 in glucose-induced GLP-1 secretion remain controversial.

Canagliflozin, a first-in-class SGLT2 inhibitor with potent antihyperglycemic activity (Nomura et al., 2009). CGMI is reported to have more potent inhibitory activity against SGLT1 than canagliflozin (Nomura et al., 2009). A recent clinical study of canagliflozin found delayed intestinal glucose absorption accompanied by increased plasma GLP-1 levels in healthy subjects (Polidori et al., 2013). Furthermore, the combined treatment with canagliflozin and teneligliptin, a DPP4 inhibitor, increased plasma active GLP-1 (aGLP-1) levels in diabetic rats (Oguma et al., 2015). Because plasma GLP-1 levels were not potentiated in SGLT2-deficient mice after a meal challenge (Powell et al., 2013), it has been suggested that GLP-1 elevation by canagliflozin is mediated by a mechanism other than the inhibition of SGLT2. However, the contribution of SGLT1 inhibition to increased plasma GLP-1 levels has not been directly determined in either animals or humans.

In the present study, we hypothesized that pharmacological inhibition of intestinal SGLT1 increases GLP-1 secretion and, when combined with DPP4 inhibition, the increase in plasma aGLP-1 has a therapeutic potential for T2DM. To address this hypothesis, we used three SGLT2 inhibitors: phloridzin, CGMI [3-(4-cyclopropylphenylmethyl)-1-(β-D-glucopyranosyl)-4-methylindole], and canagliflozin. A previous report showed that phloridzin reduced glucose-induced GLP-1 release (Moriya et al., 2009). CGMI is reported to have more potent inhibitory activity against SGLT1 than canagliflozin (Nomura et al., 2014). We explored the effects of these compounds on plasma GLP-1 levels and intestinal carbohydrate absorption and evaluated the combined effect of canagliflozin and sitagliptin, a DPP4 inhibitor, in normal and diabetic rodents.

Materials and Methods

Reagents and Chemicals

Canagliflozin, CGMI, sitagliptin, and voglibose were synthesized at the Medicinal Chemistry Laboratory at Mitsubishi Tanabe Pharma Corporation (Toda-shi, Saitama, Japan). Phloridzin was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from commercial sources and were of reagent or tissue-culture grade.

Cell-Based Assays

SGLTs Inhibition Assay. Expression plasmids containing human SGLTs, rat SGLTs, and mouse SGLTs were stably transfectected into Chinese hamster ovary–K1 cells. Cells were seeded into 24-well plates at a density of 4 × 10^4 cells/well and cultured for 2 days. The cells were washed with assay buffer (50 mM HEPES, 20 mM Tris base, 5 mM KCl, 1 mM MgCl_2, 1 mM CaCl_2, and 137 mM NaCl at pH 7.4) and were preincubated with the compound solutions for 10 minutes. Transport reaction was initiated by adding 0.3 or 0.5 mM AMG (α-methyl-D-glucopyranoside) (Sigma-Aldrich) in the presence of [14C]AMG (PerkinElmer, Waltham, MA) and was incubated for 120 minutes at 37°C. Radioactive counts in cells were assessed using a liquid scintillation counter (PerkinElmer). Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Facilitated Glucose Transporter Inhibition Assay. Glucose transporter (GLUT) 1 activity was assessed in L6 myoblast cells (Health Science Research Resources Bank, Osaka, Japan) (Mitsumoto et al., 1991). Cells were seeded into 24-well plates at a density of 3 × 10^5 cells/well and cultured for 24 hours. Prior to the transport experiment, the cells were washed with Krebs-Ringer-phosphate-HEPES buffer (150 mM NaCl, 5 mM KCl, 1.25 mM MgSO_4, 1.25 mM CaCl_2, 10 mM HEPES, and 2.9 mM Na_2HPO_4 at pH 7.4) and were preincubated with the compound solution for 5 minutes. Transport reaction was initiated by adding 2-deoxyglucose (Tokyo Chemical Industry, Tokyo, Japan) and [3H]2-deoxyglucose (American Radio- labeled Chemicals, St. Louis, MO) and was incubated for 30 minutes at room temperature. Incorporation radioactivity was determined using a liquid scintillation counter (PerkinElmer). Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce).

DPP4 Inhibition Assay. Inhibitory activities of test compounds against rat DPP4 were measured using serum collected from 7-week-old male Sprague-Dawley (SD) rats (Charles River Japan, Yokohama, Japan). Rat serum and Gly-Pro 4-methylcoumaryl-7-amide were mixed with the assay buffer (phosphate-buffered saline containing 0.003% Brij-35 solution) to initiate the enzyme reaction, as described previously (Fukuda-Tsuru et al., 2012). The fluorescence intensity of Gly-Pro 4-methylcoumaryl-7-amide was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) after 1-hour incubation at 37°C.

In Vivo Studies

Animals and Test Compound Administration. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation and met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments. Male C57BL/6J mice were purchased from CLEA Japan (Kanagawa, Japan). Male SD rats and Zucker diabetic fatty (ZDF) rats were purchased from Charles River Japan. During an acclimatization period of at least 1 week, the animals were housed in a temperature- and humidity-controlled environment with 12-hour light/dark cycles. They were provided water ad libitum and a standard commercial diet. Test compounds for oral gavage were prepared in 0.5% carboxymethylcellulose containing 0.2% Tween 80. The vehicle, instead of the drug, was administered in the control groups.

Oral Glucose Tolerance Test in Mice. After overnight fasting, 9-week-old C57BL/6J mice were simultaneously treated with test compounds and glucose solution at 2 g/kg body weight. Blood was collected from the abdominal aorta into chilled tubes containing EDTA (Djingo Laboratories, Kumamoto, Japan) and a DPP4 inhibitor (Millipore, Billerica, MA) while under anesthesia with isoflurane (Escain; Mylan, Tokyo, Japan) before and at 5, 30, 60, 120, and 180 minutes after administration. After centrifugation, plasma was stored at −80°C until the measurement of plasma aGLP-1.

Oral Glucose Tolerance Test and Fat Tolerance Test in Rats. After overnight fasting, test compounds and glucose solution (2 g/kg p.o.) were simultaneously administered to 7- to 8-week-old SD rats. Blood was collected from the tail vein before and at 10, 30, 60, 120, and 180 minutes after administration. Oral glucose tolerance tests (OGTTs) in 8- or 13-week-old ZDF rats were performed using the same protocol. In the fat tolerance test, 0.3 mg/kg of CGMI and water or a fat emulsion, 2 g/kg Intralipos (Otsuka Pharmaceutical Factory, Tokushima, Japan), instead of glucose solution were orally administered. Blood was collected into chilled tubes containing EDTA and a DPP4 inhibitor, and after centrifugation, plasma was stored at −80°C until the measurement of plasma glucose, insulin, and aGLP-1.

Sucrose Loading Test in Normal Rats. Overnight-fasted 7-week-old SD rats were orally treated with CGMI and sucrose solution (2.5 g/kg) simultaneously. Blood was collected from the tail vein at
0, 30, 60, 120, and 180 minutes after administration for plasma glucose monitoring. In another set of experiments, the rats were anesthetized with pentobarbital sodium (Tokyo Chemical Industry) (50 mg/kg i.p.) at 1 hour after administration, and the small intestine was removed and divided into three parts: upper small intestine (lower segment 20 cm from the pylorus), middle small intestine (segment between upper and lower intestine), and lower small intestine (upper segment 20 cm from the ileocecal junction). The contents of these intestinal segments were collected with 5 ml of ice-cold saline, and the volume of the contents was measured. Carbohydrates in collected samples were hydrolyzed with \( \text{H}_2\text{SO}_4 \) by boiling and neutralized with NaOH. Glucose contents were then measured. For calculating sucrose dosage, the sucrose solution was hydrolyzed, and the glucose concentration was determined in the same way.

For evaluating the effects of canagliflozin and voglibose, the rats were anesthetized 1 or 6 hours after administration and GI tracts were removed and divided into five parts: upper small intestine, middle small intestine, lower small intestine, cecum, and large intestine. Other procedures were performed in the same way as the experiment with CGMI. The intraluminal contents of canagliflozin in a portion of the fluid collected from the upper small intestine were determined by liquid chromatography–tandem mass spectrometry, API 4000 (AB SCIEX, Framingham, MA) equipped with a Cadenza CD C-18 column, 2.0-mm i.d.

**Determination of Metabolic Parameters.** Glucose concentrations in plasma and GI contents were determined using a glucose CII-test Wako kit (Wako Pure Chemical Industries, Osaka, Japan). Insulin concentrations were measured using an ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biologic Science, Yokohama, Japan). Plasma active GLP-1 was determined by an ELISA kit (Millipore or Epitope Diagnostics, San Diego, CA) after solid phase extraction with an Oasis HLB μElution plate (Waters, Milford, MA) (Franz and Li, 2012; Kinoshita and Kondo, 2015). Plasma total GLP-1 was determined by an ELISA kit (Meso Scale Discovery, Rockville, MD). The baseline values are defined as plasma GLP-1 concentrations at 0 hours in the vehicle-treated group, and plasma GLP-1 relative levels were normalized and expressed as the ratio to the baseline in each figure. aGLP-1 baseline values ranged from 0.10 to 1.17 pmol/l in all experiments, and the total GLP-1 baseline value was 0.62 pmol/l.

**Statistical Analysis.** Data in all figures were presented as the arithmetic means ± S.E.M. for each group, and IC\(_50\) values in Tables 1 and 2 were expressed as the geometric mean of two to three experiments (Fleming et al., 1972; Stórustovu and Ebert, 2006; Kuriyama et al., 2014). The area under the curve (AUC) and incremental AUC (ΔAUC, defined as the AUC occurring above the baseline value) were calculated by the trapezoidal rule. Statistical differences between vehicle and single treatment groups or between single and combination treatment groups were determined by one- or two-way analysis of variance followed by Student’s t test (Hwee et al., 2015; Wang et al., 2015), unless otherwise indicated in figure legends. Statistical analyses were performed using a SAS-based system (SAS Institute, Cary, NC) or Prism software (GraphPad Software, San Diego, CA). Differences assessed by each test were considered statistically significant at \( P < 0.05 \).

**Results**

**SGLT1 Inhibition and Selectivity.** Tables 1 and 2 summarize the effect of the compounds used in this study on the activities of SGLTs, GLUTs, and DPP4. Similar to phloridzin, CGMI and canagliflozin inhibited human, rat, and mouse SGLT1 (Table 1). These compounds also inhibited SGLT2 activity, and CGMI was the most potent SGLT1 inhibitor among the three compounds. These SGLT inhibitors did not inhibit GLUT1 and DPP4 activity, and sitagliptin potently inhibited DPP4 activity (Table 2).

**Effects of Phloridzin and Canagliflozin in Combination with Sitagliptin on Plasma aGLP-1 Levels in Normal Mice and Rats.** To determine whether oral treatment of phloridzin and canagliflozin affects GLP-1 secretion, we analyzed the time course of plasma GLP-1 levels during OGTT. For this purpose, the in vivo effects of these inhibitors were examined in combination with sitagliptin to prevent aGLP-1 degradation. In normoglycemic mice and rats, plasma aGLP-1 levels were not significantly changed after oral glucose loading. In mice treated with 10 mg/kg sitagliptin, the plasma aGLP-1 level was increased at 5 minutes after glucose loading (Fig. 1A). In contrast, in mice treated with 500 mg/kg phloridzin, the plasma aGLP-1 level was unchanged at 5 minutes after glucose loading, but increased at 30 minutes and thereafter. Moreover, combined treatment of phloridzin and sitagliptin increased and prolonged plasma aGLP-1 elevation compared with either monotherapy at 30 minutes and later following glucose loading. Similar results were obtained in rats (Fig. 1, B and C).

The combined treatment of canagliflozin (0.3–30 mg/kg) and sitagliptin (10 mg/kg) also increased plasma aGLP-1 levels in a dose-dependent manner in normoglycemic mice and rats (Fig. 2). Monotherapy with canagliflozin increased plasma total GLP-1 at 30 minutes after glucose loading in normoglycemic rats (Fig. 3A), and canagliflozin did not affect plasma DPP4 activity in combination with or without sitagliptin at 30 minutes after the oral administration of compounds (Fig. 3B). These data suggest that canagliflozin does not prevent inactivation but enhances GLP-1 secretion.

**TABLE 1**

Inhibitory effects of SGLT inhibitors on SGLT1 and SGLT2.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_50) Human SGLT1</th>
<th>IC(_50) Human SGLT2</th>
<th>IC(_50) Rat SGLT1</th>
<th>IC(_50) Rat SGLT2</th>
<th>IC(_50) Mouse SGLT1</th>
<th>IC(_50) Mouse SGLT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloridzin</td>
<td>241</td>
<td>23.8</td>
<td>609</td>
<td>49.1</td>
<td>760</td>
<td>65.6</td>
</tr>
<tr>
<td>CGMI</td>
<td>22.1</td>
<td>1.39</td>
<td>39.4</td>
<td>2.73</td>
<td>41.5</td>
<td>6.31</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>663</td>
<td>4.2</td>
<td>555</td>
<td>3.7</td>
<td>613</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Data from Kuriyama et al. (2014).*

**TABLE 2**

Inhibitory effects of SGLT inhibitors and sitagliptin on rat GLUT and DPP4.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_50) GLUT</th>
<th>IC(_50) DPP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloridzin</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>CGMI</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>N.D.</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*Data from Kuriyama et al. (2014).*
Effects of CGMI and Canagliflozin in Combination with Sitagliptin on Plasma GLP-1 Levels in Normal Rats. Subsequently, we analyzed the involvement of SGLT1 in GLP-1 secretion in detail using CGMI and canagliflozin, which are potent and weak SGLT1 inhibitors, respectively. In sitagliptin-treated rats, glucose loading increased plasma GLP-1 concentrations in a manner dependent on the dose of CGMI (0.1–1 mg/kg) (Fig. 4A). The efficacy of 0.3 mg/kg CGMI in increasing plasma GLP-1 levels was almost equivalent to that of 10 mg/kg canagliflozin (Fig. 4B). This dose ratio of CGMI and canagliflozin was consistent with the inhibitory activities of these compounds against SGLT1.

To further investigate whether intestinal SGLT1 inhibition contributes to elevated plasma GLP-1, we compared the stimulant for GLP-1 secretion, glucose as SGLT1 substrate and water or fat as a non-SGLT1 substrate. Oral water loading did not increase plasma GLP-1 levels in rats treated with sitagliptin alone and with combination CGMI and canagliflozin.
Fig. 3. Effects of canagliflozin on plasma total GLP-1 levels and DPP4 activity during OGTT in SD rats. (A) Plasma total GLP-1 relative levels at 0 and 30 minutes after glucose administration in canagliflozin (0.3–30 mg/kg) treated rats. (B) Plasma DPP4 activity at 0 and 30 minutes after glucose administration in rats. Canagliflozin, sitagliptin, and glucose solution (2 g/kg) were simultaneously administered to SD rats at time 0 by oral gavage. Blood was collected from the tail vein in rats. Data are presented as the mean ± S.E.M. (N = 6). **P < 0.01 versus vehicle group by one-way analysis of variance with Dunnett’s post hoc test. In plasma DPP4 activity, there were no significant differences between the canagliflozin (1–10 mg/kg) versus vehicle group and between the sitagliptin + canagliflozin (1–10 mg/kg) versus sitagliptin group by one-way analysis of variance with Dunnett’s post hoc test, respectively.

Fig. 4. Effects of CGMI combined with sitagliptin on plasma aGLP-1 levels during OGTT in SD rats. (A and B) Time course and AUC<sub>0–3 hr</sub> of plasma aGLP-1 relative levels. CGMI (0.1–1 mg/kg), canagliflozin (10 mg/kg), sitagliptin (10 mg/kg), and glucose solution (2 g/kg) were administered to SD rats at time 0 by oral gavage, and blood was collected from the tail vein. Data are presented as the mean ± S.E.M. (N = 6). *P < 0.05 versus vehicle group; **P < 0.01 versus sitagliptin group; and ***P < 0.01 versus sitagliptin group by one-way analysis of variance with Dunnett’s post hoc test.
Fig. 5. Effects of CGMI combined with sitagliptin on plasma aGLP-1 levels during oral glucose or fat tolerance test in SD rats. (A and B) Time course and AUC_{0-3h} of plasma aGLP-1 relative levels after glucose or water loading. (C and D) Time course and AUC_{0-3h} of plasma aGLP-1 relative levels after glucose or fat loading. CGMI (0.3 mg/kg), sitagliptin (10 mg/kg), and glucose solution (2 g/kg) were administered to SD rats at time 0 by oral gavage, and blood was collected from the tail vein. Data are presented as the mean ± S.E.M. (N = 6). **P < 0.01 versus vehicle-treated glucose-loading group; ##P < 0.01 versus sitagliptin-treated glucose-loading group; $$$P < 0.01 versus vehicle-treated fat-loading group.
Effect of CGMI, Canagliflozin, and Voglibose on Intestinal Carbohydrate Absorption in Rats. As only glucose-induced aGLP-1 increase was enhanced by the combination of CGMI and a DPP4 inhibitor, we then analyzed the effects of CGMI, canagliflozin, and voglibose on intestinal carbohydrate absorption in a sucrose loading test. Voglibose, an α-glucosidase inhibitor, was used as a positive control to validate the experiment of intestinal carbohydrate contents (Bischoff, 1994; Goke et al., 1994). Fasted SD rats were orally treated with 0.3 mg/kg CGMI, 10 mg/kg canagliflozin, or 0.1 mg/kg voglibose and sucrose (2.5 g/kg) simultaneously. CGMI significantly increased the residual carbohydrate contents in the middle and lower parts of the small intestine at 1 hour after sucrose administration (Fig. 6A). Although peak plasma glucose concentrations following sucrose loading were suppressed, the AUC<sub>0–3</sub> hours of plasma glucose was not affected by CGMI treatment (Fig. 6, B and C).

Similar to CGMI, both canagliflozin and voglibose significantly increased residual carbohydrate contents in the upper and middle parts of the small intestine at 1 hour after sucrose loading (Fig. 7A). Voglibose increased cecal carbohydrate contents at 6 hours after sucrose loading, indicating sustained inhibition of carbohydrate absorption (Fig. 7B). Canagliflozin, however, did not increase residual carbohydrate contents at 6 hours after sucrose loading. The intraluminal amount of canagliflozin in the upper small intestine at 1 and 6 hours after administration was 93.86 ± 64.81 and 16.11 ± 11.87 nmol, respectively. Considering that the water volume in the rat whole small intestine is 11.1 g/kg body weight (McConnell et al., 2008), the intraluminal concentration of canagliflozin would be 35.4 and 6.2 μmol/l at 1 and 6 hours, respectively, after administration in the upper small intestine. Intraluminal canagliflozin concentration was estimated to be more than 10 times higher than the IC<sub>50</sub> (555 nmol/l) for rat SGLT1 inhibition. Therefore, it is suggested that canagliflozin and CGMI transiently inhibited and delayed intestinal carbohydrate absorption in contrast to the sustained inhibition induced by an α-glucosidase inhibitor.

Effect of Canagliflozin in Combination with Sitagliptin on Plasma aGLP-1 Levels during OGTT in ZDF Rats. ZDF rats rapidly progress from normoglycemia to frank diabetes within 12 weeks of age and are ultimately characterized by extensive insulin resistance, hyperinsulinemia, hyperglycemia, and hyperlipidemia similar to obese patients with T2DM (Szocs et al., 2008; Watanabe et al., 2015). In 8-week-old ZDF rats, in which the fasting blood glucose concentration is not increased, the plasma aGLP-1 level was increased in canagliflozin- and sitagliptin-treated groups and was further augmented in the combined treatment group (Fig. 8, E and F). Inhibition of DPP4 by sitagliptin increased insulin levels and reduced plasma glucose levels induced by oral glucose (Fig. 8, A–D). Additional canagliflozin treatment further reduced the plasma glucose excursion in OGTT. In 13-week-old ZDF rats, in which the fasting blood glucose level is extensively increased, sitagliptin increased both plasma aGLP-1 and insulin levels and reduced plasma glucose levels after glucose loading (Fig. 9). Additional treatment of canagliflozin in combination with sitagliptin treatment further increased the aGLP-1 level and extensively reduced the increase of plasma glucose excursion. The effect of
combination treatment on plasma aGLP-1 and glucose levels was greater than that of the canagliflozin single treatment.

**Discussion**

Although SGLT1 was the first molecule identified within the SGLT family and its role of dietary glucose absorption has been studied well (Kanai et al., 1994b, 1995), the role of SGLT1 on incretin secretion in vivo is still controversial. It has been demonstrated that SGLT1 is crucial for incretin secretion because phloridzin blocked the increase in portal aGLP-1 levels immediately after glucose administration into the small intestine (Moriya et al., 2009). Furthermore, in Sglt1−/− mice, plasma GLP-1 levels at 5 minutes after oral glucose challenge are reduced and glucose fails to stimulate incretin secretion from intestinal cultures in vitro (Gorboulev et al., 2012). In these previous studies, however, GLP-1 levels were only measured in an early phase at 5 minutes after glucose loading, but not in delayed phases at 30 minutes and later. In the present study, phloridzin did not suppress plasma aGLP-1 levels at 5 minutes, but rather enhanced them at 30 minutes and later after glucose loading in normoglycemic mice, especially in combination with sitagliptin. Similarly, CGMI and canagliflozin, an SGLT2 inhibitor with a moderate inhibitory activity against SGLT1, enhanced GLP-1 secretion and increased plasma aGLP-1 levels in combination with sitagliptin in glucose-loaded SD rats without affecting basal plasma aGLP-1 levels. Because plasma GLP-1 levels were not potentiated in SGLT2-deficient mice after a meal challenge (Powell et al., 2013), plasma aGLP-1 increase by phloridzin, CGMI, and canagliflozin would be attributed to SGLT1 inhibition.

CGMI, a potent SGLT1 inhibitor, increased glucose-induced, but not fat-induced, plasma aGLP-1 increase at a lower dose compared with canagliflozin. Moreover, the residual carbohydrate contents after oral sucrose ingestion were increased in the middle and lower part of small intestine and the peak plasma glucose concentration was delayed in animals orally treated with CGMI. Total absorption of carbohydrates was not inhibited, as the AUC of plasma glucose levels following oral sucrose challenge was not reduced by CGMI. Therefore, the results indicate that intestinal SGLT1 inhibition suppresses carbohydrate absorption in the upper segment of the GI tract and increases delivery to the lower segment. Because glucose is a representative stimulant of GLP-1 secretion, it is likely that nonabsorbed glucose

![Fig. 7. Effects of canagliflozin on carbohydrate absorption in the GI tract in sucrose-loaded SD rats. (A and B) Carbohydrate content in the upper small intestine, middle small intestine, lower small intestine, cecum, large intestine, and total segment of intestine 1 hour (A) or 6 hours (B) after sucrose loading. Canagliflozin (10 mg/kg) or voglibose (0.1 mg/kg) and sucrose solution (2.5 g/kg) were simultaneously administered to SD rats by oral gavage. Intestinal contents were collected from the GI tract divided into five parts. Data are presented as the mean ± S.E.M. (N = 4–6). *P < 0.05 and **P < 0.01 versus vehicle group.](image-url)
delivered into the lower segment facilitates the release of GLP-1 from L cells, which are abundantly located in the lower part of the small intestine.

CGMI and canagliflozin transiently inhibited carbohydrate absorption in the upper small intestine after oral administration. Although SGLT1 is abundantly present in the intestine and plays a critical role in intestinal glucose absorption (Gorboulev et al., 2012), SGLT2 is rarely expressed in the intestine (Chen et al., 2010). Therefore, it is suggested that the effects of these compounds are mediated mainly by inhibition of SGLT1. A previous study (Kuriyama et al., 2014) has shown that the plasma $C_{\text{max}}$ level of canagliflozin following oral administration is below the IC$_{50}$ value for SGLT1; therefore, it is unlikely that canagliflozin inhibits intestinal SGLT1 as a systemic effect. Although it is difficult to determine the precise concentration of canagliflozin in the upper small intestine from the remaining intraluminal content, the estimated concentration markedly exceeded the IC$_{50}$ value for SGLT1. The concentrations of CGMI and canagliflozin were transiently high enough to inhibit SGLT1 and delay the absorption of glucose in the upper small intestine after oral administration. It is suggested that transient inhibition of SGLT1 in the small intestine delayed the absorption of glucose and the nonabsorbed glucose delivered to the lower small intestine augmented GLP-1 secretion in the L cells.

Sustained inhibition of carbohydrate absorption in the small intestine is accompanied by severe intestinal symptoms, as shown in the phenotype of SGLT1 deficiency (Gorboulev et al., 2012) and adverse effects of $\alpha$-glucosidase inhibitors (Goke et al., 1994; Martin and Montgomery, 1996; Scott and Spencer, 2000). Systemic inhibition of SGLT1 suppresses glucose absorption in the entire GI tract, and nonabsorbed glucose delivered into the cecum and colon facilitates microbial fermentation and production of short chain fatty acids, which in turn activate GLP-1 secretion from L cells (Gorboulev et al., 2012; Powell et al., 2013). In contrast to the sustained inhibition of carbohydrate absorption in the entire digestive tract by agents, such as the $\alpha$-glucosidase inhibitor, transient inhibition of SGLT1 only delayed glucose absorption and enhanced GLP-1 secretion without increasing residual carbohydrate contents in the cecum and colon. This suggests that transient and moderate SGLT1 inhibition leads to GLP-1 release without adverse effects in digestive systems.

Because SGLT1 is abundantly expressed in the duodenum in patients with T2DM (Freitas et al., 2008; Gerich, 2010) and aGLP-1 increases nutrient-induced insulin release (Nauck et al., 1997; Gromada et al., 1998), we determined the potential of concomitant inhibition of SGLT1 and DPP4 in hyperglycemic animals. In ZDF rats, an animal model exhibiting progressive diabetes similar to human T2DM,
insulin resistance develops at 7 weeks of age and plasma glucose levels increase by 13 weeks of age (Szocs et al., 2008; Watanabe et al., 2015). Plasma aGLP-1 levels in ZDF rats were higher after combination treatment with canagliflozin and sitagliptin compared with monotherapy of either drug at both 8 and 13 weeks of age. In sitagliptin-treated ZDF rats at both early and middle stages of diabetes, canagliflozin treatment enhanced the increase in plasma aGLP-1 levels and further reduced plasma glucose excursion during OGTT. These antihyperglycemic effects are likely to be mediated by the enhancement of GLP-1 secretion and the delay of glucose absorption because of intestinal SGLT1 inhibition in addition to the suppression of renal glucose reabsorption by SGLT2 inhibition. Although glucose response in OGTT was markedly suppressed, the plasma insulin response in the combined treatment group was not significantly different from that in the sitagliptin-treated group. It is likely that increased plasma aGLP-1 enhances glucose-induced insulin release in OGTT in spite of suppressed glucose levels. These results suggest that concomitant inhibition of SGLT1 and DPP4 increases plasma aGLP-1 levels and improves glucose tolerance in hyperglycemia.

In summary, the present study demonstrates that transient and moderate inhibition of SGLT1 in the proximal part of the intestine increases the release of GLP-1 through increasing glucose concentrations in the distal part of the small intestine. Concomitant inhibition of SGLT1 and DPP4 is a novel therapeutic option for the improvement of glucose tolerance and hyperglycemia in T2DM.

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