Selective SGLT2 inhibition by tofogliflozin reduces renal glucose reabsorption under hyperglycemic but not under hypo- or euglycemic conditions in rats

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Am J Physiol Endocrinol Metab 304: E414–E423, 2013. First published December 18, 2012; doi:10.1152/ajpendo.00545.2012.—To understand the risk of hypoglycemia associated with urinary glucose excretion (UGE) induced by sodium-glucose cotransporter (SGLT) inhibitors, it is necessary to know the relationship between the ratio of contribution of SGLT2 vs. SGLT1 to renal glucose reabsorption (RGR) and the glycemic levels in vivo. To examine the contributions of SGLT2 and SGLT1 in normal rats, we compared the RGR inhibition by tofogliflozin, a highly specific SGLT2 inhibitor, and phlorizin, an SGLT1 and SGLT2 (SGLT1/2) inhibitor, at plasma concentrations sufficient to completely inhibit rat SGLT2 (rSGLT2) while inhibiting rSGLT1 to different degrees. Under hyperglycemic conditions by glucose titration, tofogliflozin and phlorizin achieved ≥50% inhibition of RGR. Under hypoglycemic conditions by hyperinsulinemic clamp, RGR was reduced by 20–50% with phlorizin and by 1–5% with tofogliflozin, suggesting the smaller contribution of rSGLT2 to RGR under hypoglycemic conditions than under hyperglycemic conditions. Next, to evaluate the hypoglycemic potentials of SGLT1/2 inhibition, we measured the plasma glucose (PG) and endogenous glucose production (EGP) simultaneously after UGE induction by SGLT inhibitors. Tofogliflozin (400 ng/ml) induced UGE of about 2 mg·kg\(^{-1}\)·min\(^{-1}\) and increased EGP by 1–2 mg·kg\(^{-1}\)·min\(^{-1}\), resulting in PG in the normal range. Phlorizin (1,333 ng/ml) induced UGE of about 6 mg·kg\(^{-1}\)·min\(^{-1}\) and increased EGP by about 4 mg·kg\(^{-1}\)·min\(^{-1}\); this was more than with tofogliflozin, but the minimum PG was lower. These results suggest that the contribution of SGLT1 to RGR is greater under lower glycemic conditions than under hyperglycemic conditions and that SGLT2-selective inhibitors pose a lower risk of hypoglycemia than SGLT1/2 inhibitors.

RENAI GLUCOSE REABSORPTION (RGR) is mediated by sodium-glucose cotransporters, namely the high-affinity sodium-glucose cotransporter SGLT1 (SLC5A1) and the low-affinity sodium-glucose cotransporter SGLT2 (SLC5A2), in the proximal tubules (25). Recently, several SGLT inhibitors have been developed for the treatment of type 2 diabetes (3, 6). Although SGLT1 is reported to be expressed in several organs, such as the intestine, kidney, brain, and heart, SGLT2 is predominantly distributed in the kidney (5, 25). Therefore, because of the concern associated with inhibiting SGLT1 in these organs, it is reasonable that most of the SGLT inhibitors being currently evaluated are SGLT2-specific.

It had been considered that SGLT2 contributes about 90% and SGLT1 contributes about 10% to RGR (3, 25), suggesting that inhibition of renal SGLT1 may have a negligible impact on RGR. However, a recent electrophysiological study using a whole cell patch-clamp system showed that K\(_{0.5}\) (glucose) of human SGLT2 (hSGLT2) (4.9 mM) is greater than that of hSGLT1 (1.8 mM), suggesting that hSGLT2 works at only 50% capacity under euglycemic conditions, whereas hSGLT1 would work more dominantly under hypoglycemic conditions (9). These findings are consistent with the percentage inhibition of RGR found in clinical studies with dapagliflozin, a relatively SGLT2-specific inhibitor that was calculated to be at a maximum of about 50% even at plasma concentrations that were expected to completely inhibit hSGLT2 (11, 13, 14). In addition, SGLT2 knockout mice showed relatively higher (up to 60%) fractional reabsorption of glucose in the proximal tubules at lower rates of glucose filtration, suggesting that SGLT1 or another glucose transporter may have a higher capacity to reabsorb glucose under euglycemic conditions (23, 24). These findings imply that the relative contribution of SGLT1 to RGR compared with that of SGLT2 may be greater under hypo- or euglycemic conditions than under hyperglycemic conditions. If this is the case, the selectivity of SGLT inhibitors to SGLT2 vs. selectivity to SGLT1 can be critical to determining the hypoglycemic potentials of SGLT inhibitors. Therefore, it is important to determine the relationship between glucose levels in vivo and the ratio of contribution of SGLT1 vs. SGLT2.

One way to more accurately understand the contributions of SGLT1 and SGLT2 to RGR in vivo is to evaluate the efficacy of inhibitors specific to certain SGLTs under variable glycemic conditions.

Tofogliflozin (17) is a highly specific SGLT2 inhibitor [IC\(_{50}\) values against rat SGLT1 (rSGLT1) and rSGLT2 are 8,200 and 15 nM, respectively (21)] currently under clinical development (10). In diabetic animal models, such as Zucker diabetic fatty rats, this compound had blood glucose-lowering effects accompanied with increased urinary glucose excretion (UGE); on the other hand, it showed no significant hypoglycemic effects in normal rats (21). Phlorizin is an SGLT inhibitor that has shown inhibitory activities against several SGLTs in addition to SGLT1 and SGLT2 (21); however, the contribution of these

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SGLTs to RGR is considered minimal compared with the contributions of SGLT1 and SGLT2 (25). Therefore, to understand the contribution of SGLT1 in RGR, it is feasible to compare the efficacies of tofogliflozin with those of phlorizin as an SGLT1/2 inhibitor (IC50 values for phlorizin against rSGLT1 and rSGLT2 are 970 and 48 nM, respectively) (21). It should be noted that this protocol necessitates stably controlled plasma glucose levels and glomerular filtration rate (GFR), since UGE levels are influenced by plasma glucose levels and GFR (12, 26).

In addition, if SGLT1 has a greater role in glucose reabsorption under hypo- and euclidean conditions, it also implies that the inhibition of SGLT1 under these conditions may exacerbate hypoglycemia via induction of excessive UGE compared with selective SGLT2 inhibition.

In a previous study, repeated subcutaneous injection of phlorizin in streptozotocin-induced diabetic rats showed no severe hypoglycemic effect (19); however, the lack of pharmacokinetic data in that study makes interpretation of the results difficult. In addition, in clinical trials of SGLT2 inhibitors, no significant increases in hypoglycemic episodes were reported under euclidean conditions in healthy volunteers despite the induction of UGE (13, 20), which is explained as counterregulation by endogenous glucose production (EGP) accompanying the induction of UGE (13). However, no detailed studies are available on the relationships between UGE and EGP. To examine the hypoglycemic potentials of UGE with SGLT inhibitors, it is important to evaluate UGE and EGP simultaneously.

In this study, we compared the inhibitory effects of tofogliflozin and phlorizin on RGR in rats under variable glycemic conditions. In particular, we tried to fix the plasma concentrations of each of the two compounds at a constant level that can completely inhibit rSGLT2 and may inhibit rSGLT1 to a certain extent with controlled stable plasma glucose levels and GFR. In addition, we evaluated the EGP and UGE induced by tofogliflozin or phlorizin under euclidean conditions.

MATERIALS AND METHODS

Chemicals

Tofogliflozin [(1S,3R,4’S,5’S,6’S)-6-{(4-ethylphenyl)methyl}-3’,4’,5’,6’-tetrahydro-6’-(hydroxymethyl)-spiro{isobenzofuran-1(3H),2’,2’-[2H]pyran]-3’,4’,5’,triol] (17) was synthesized in our laboratory at Chugai Pharmaceutical. Phlorizin was purchased from Sigma-Aldrich (St. Louis, MO). Insulin (Novolin R; 100 U/ml) was purchased from Novo Nordisk Pharma (Tokyo, Japan). Uniformly labeled [U-13C]glucose (99 atom percent excess) was purchased from Cambridge Isotope Laboratories (Andover, MA). Glucose solutions of 20 and 50% were purchased from FUSO Pharmaceutical Industries (Osaka, Japan) and from Otsuka Pharmaceutical Factory (Tokushima, Japan), respectively. Tofogliflozin was dissolved at 0.6 mg/ml in saline and diluted serially. Phlorizin was dissolved at 0.2 mg/ml in saline and diluted serially. Insulin was diluted at 2 U/ml with saline. Glucose solutions (20 or 50%) were diluted with purified water to make concentrations of 10, 30, or 40%.

Animals

Male Wistar rats (Jcl:Wistar) were purchased from CLEA Japan (Tokyo, Japan). These animals were housed under a 12:12-h light-dark cycle (lights on 7:00 A.M.-7:00 P.M.) with controlled room temperature (20–26°C) and humidity (35–75%) and were allowed ad libitum access to a diet of laboratory chow (CE-2 pellets; Clea Japan) and water. All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical. The protocol was approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical.

Surgical Operation

Rats at 9–12 wk of age, weighing 260–370 g, were anesthetized with an intraperitoneal injection of thiobutabarbital sodium salt (120 mg/kg) and placed on a heating pad to maintain body temperature at 36–38°C. The trachea was cannulated with polyethylene tubing for breathing. For infusion of drugs (SGLT inhibitors and insulin) and glucose, the right femoral vein was cannulated with three PE-10 polyethylene catheters (V1–V3 in the clamp study) or two PE-10 polyethylene catheters (V1–V2 in the titration and infusion study). The right carotid artery was cannulated with PE-50 polyethylene catheters for monitoring blood pressure and heart rate. A silicon tube catheter (ID = 1 mm) was inserted in the urinary bladder for collection of urine. Upon completion of the surgical operation, saline (15 ml/kg) was injected subcutaneously, and the animals were stabilized for 30 min.

Infusion Protocols with Blood and Urine Collection

UGE under hyperglycemic conditions induced by glucose titration (protocol 1). Each animal was infused with saline at a rate of 15 ml·kg⁻¹·h⁻¹ through vein catheter V1 and 10 ml·kg⁻¹·h⁻¹ through vein catheter V2 for 60 min. Next, the infusion of tofogliflozin or phlorizin solution was started at a rate of 2 ml/kg (bolus) plus 15 ml·kg⁻¹·h⁻¹ through vein catheter V2 without changing the constant infusion of saline at 10 ml·kg⁻¹·h⁻¹ through vein catheter V2. The concentrations of the tofogliflozin and phlorizin solutions used were determined on the basis of pharmacokinetic parameters obtained from separate pharmacokinetic studies (data not shown) to maintain plasma concentrations of 4, 13.3, 40, 133, or 400 ng/ml for tofogliflozin and 40, 133, 400, or 1,333 ng/ml for phlorizin. Namely, the infusion rate needed to achieve a target plasma concentration of tofogliflozin of 400 ng/ml was 1.2 mg/kg (bolus) and 0.5 mg·kg⁻¹·h⁻¹ (constant), and that to achieve a target plasma concentration of phlorizin of 1,333 ng/ml was 0.15 mg/kg (bolus) and 2.8 mg·kg⁻¹·h⁻¹ (constant). After 60 min of tofogliflozin or phlorizin infusion, infusion of glucose solutions (10, 20, 30, 40, and 50%) was started at 10 ml·kg⁻¹·min⁻¹ in a stepwise manner from 10% at 30-min intervals through vein catheter V2 to raise the plasma glucose concentration to above 400 mg/dl. A blood sample (0.25 ml) was collected every 15 min with a heparinized syringe; the plasma glucose level in the sample was checked with a plasma glucose monitoring system (Accu-check Aviva; Roche Diagnostic, Tokyo, Japan), and then a plasma sample was obtained by centrifugation to determine plasma glucose and creatinine levels and tofogliflozin or phlorizin concentrations. Urine was collected at 30-min intervals after glucose infusion to weigh polyethylene sample tubes through the bladder catheter. The catheter was flushed with 0.5 ml saline to minimize the residual urine. Urine volume was determined by subtracting the weight of the weighed sample tube from the sampled urine plus tube weight, with the specific gravity of sampled urine as 1. Urine and plasma samples were stored at −80°C until use.

UGE under hypo- and euclidean conditions induced by glucose clamp (protocol 2). Each animal was infused with saline at the rate of 15 ml·kg⁻¹·h⁻¹ through vein catheter V1 and 10 ml·kg⁻¹·h⁻¹ through vein catheter V2 for 90 min. Next, insulin (40 mU·kg⁻¹·min⁻¹ for 3 min; 20 mU·kg⁻¹·min⁻¹, constant) infusion was started through vein catheter V2. After 30 min of insulin infusion, infusion of tofogliflozin or phlorizin solution was started at a rate of 2 ml/kg (bolus) and 15 ml·kg⁻¹·h⁻¹ (constant) through vein catheter V1 without changing the constant infusion of saline at 10 ml·kg⁻¹·h⁻¹ through vein catheter
V2. The concentrations of tofogliflozin and phlorizin solution used were determined as in protocol 1. After infusion of tofogliflozin or phlorizin solution for 60 min, glucose (20%) infusion was started through vein catheter V2 at a variable infusion rate based on a formula calculated to raise the plasma concentration to around 100 mg/dl (7). After this glucose infusion, blood (0.01 ml) was sampled from the jugular vein every 5–10 min, the plasma glucose levels were measured using Accu-check Aviva, and the glucose infusion rate was adjusted based on the same formula (7). Additional blood samples (0.25 ml) and urine samples were collected and prepared in the same manner as protocol 1. In this protocol, we defined UGE under hypoglycemic conditions as that during the last 30 min of the insulin plus tofogliflozin or insulin plus phlorizin infusion period and the UGE under euglycemic conditions as that during the last 30 min of insulin plus tofogliflozin or phlorizin with glucose infusion as indicated in Fig. 4.

Effects of acute UGE induced by tofogliflozin or phlorizin on plasma glucose levels and EGP (protocol 3). Each animal was infused with saline at the rate of 25 ml·kg⁻¹·h⁻¹ through vein catheter V1 and [U-13C]glucose (99%) saline solution at 0.14 mg·kg⁻¹·min⁻¹ through vein catheter V2. After a basal infusion period of 150 min, infusion of tofogliflozin (bolus, 1.2 mg/kg; constant, 0.5 mg·kg⁻¹·h⁻¹) or phlorizin (bolus, 0.15 mg/kg; constant, 2.8 mg·kg⁻¹·h⁻¹) was started at the rate of 2 ml/kg (bolus) and 25 ml·kg⁻¹·h⁻¹ (constant) through vein catheter V1. Blood and urine samples were collected and prepared in the same manner as protocol 1 for 120 min from the start of tofogliflozin or phlorizin infusion.

Analysis

Plasma tofogliflozin concentrations were measured with a HPLC-MS/MS system [Shimadzu 20A (Shimadzu, Kyoto, Japan), API-4000 (AB SCIEX, Framingham, MA)]. Plasma phlorizin concentrations were measured with a HPLC-MS/MS system [ACQUITY UPLC Waters, Milford, MA], API-3200 (AB SCIEX)]. Blood and urinary glucose concentrations were measured by the hexokinase glucose-6-phosphate dehydrogenase method (L-Type Glu 2; Wako Pure Chemical Industries, Osaka, Japan) with an automated analyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan). Creatinine concentrations of plasma and urine were measured by the creatinase-HMMPS method (L-Type Creatinine M; Wako Pure Chemical Industries) with the automated analyzer.

Plasma [U-13C]glucose concentrations, together with that of an internal standard (fructose), were determined with a HPLC-MS/MS system [Shimadzu 20A (Shimadzu), API-4000 (AB SCIEX)] with an improved procedure to increase the sensitivity by Cs⁺ attachment to the sugars (18).

Calculations

In protocols 1 and 2, the following parameters were calculated:

\[
\text{Creatinine clearance (ml/min) = \frac{\text{urine creatinine (mg/dl)}}{\text{urine excretion rate (ml/min)/plasma creatinine (mg/dl)\times 100}}}
\]

\[
\text{UGE (mg/min) = \frac{\text{urine glucose (mg/dl)}}{\text{urine excretion rate (ml/min)/plasma glucose (mg/dl)}}}
\]

Glucose clearance (ml/min) = plasma glucose concentration × 100 (%)

Percentage inhibition of RGR [RGR inhibition (%)] = glucose clearance/creatinine clearance × 100 (%)

This calculation method was based on formulas used in a clinical study (11). In protocol 1, an apparent increase in UGE was observed with vehicle alone when plasma glucose was above around 300 mg/dl, which may result in the overestimation of percentage RGR inhibition; therefore, we defined the percentage inhibition of RGR in protocol 1 as the glucose clearance/creatinine clearance × 100 (%) when the plasma glucose levels were within 250–350 mg/dl for each rat.

In protocol 3, the rate of EGP was calculated according to the following equation (2)

\[
\text{EGP (Rₚ) = f \times \left[ \left(\frac{\text{IE}_{\text{infusate}}}{\text{IE}_{\text{plasma}}} \right) - 1 \right]}
\]

where Rₚ is the rate of glucose production, f is infusion rate of [U-13C]glucose, IEinfusate is isotopic enrichment of [U-13C]glucose in infused, and IEplasma is isotopic enrichment (%) of [U-13C]glucose in plasma (= plasma [U-13C]glucose concentration/total plasma glucose concentration × 100).

Statistical Analysis

Data are presented as means ± SD. Statistical analysis was performed with SAS System for Windows, Release 8.02 (SAS Institute Japan, Tokyo, Japan). Statistical significance was determined by the parametric Dunnett’s multiple-comparison or Student’s (unpaired) t-test.

RESULTS

UGE under Hyperglycemic Conditions Induced by Glucose Titration

The plasma concentrations of tofogliflozin and phlorizin in the glucose titration protocol are shown in Table 1. The means

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Plasma Concentration, ng/ml</th>
<th>n</th>
<th>Basal</th>
<th>Maximum % inhibition of RGR*</th>
<th>Actual Plasma Concentration of SGLT Inhibitor, ng/ml</th>
<th>Time after the Start of Infusion, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>135</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tofogliflozin</td>
<td></td>
<td>4</td>
<td>120 ± 9</td>
<td>300 ± 26</td>
<td>5.0 ± 0.5</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>116 ± 9</td>
<td>312 ± 31</td>
<td>17.0 ± 0.7</td>
<td>13.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>119 ± 5</td>
<td>319 ± 31</td>
<td>57.9 ± 2.6</td>
<td>43.8 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>133</td>
<td>114 ± 8</td>
<td>325 ± 33</td>
<td>196.9 ± 9.1</td>
<td>153.1 ± 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>111 ± 6</td>
<td>321 ± 36</td>
<td>570.1 ± 34.7</td>
<td>446.0 ± 40.1</td>
</tr>
<tr>
<td>Phlorizin</td>
<td></td>
<td>40</td>
<td>127 ± 11</td>
<td>312 ± 18</td>
<td>40.2 ± 10.5</td>
<td>44.4 ± 4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>133</td>
<td>121 ± 4</td>
<td>299 ± 17</td>
<td>145.0 ± 13.1</td>
<td>167.0 ± 13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>127 ± 4</td>
<td>303 ± 18</td>
<td>436.0 ± 24.0</td>
<td>434.7 ± 29.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,333</td>
<td>127 ± 8</td>
<td>290 ± 19</td>
<td>1,593.3 ± 83.3</td>
<td>1,463.3 ± 228.1</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats. SGLT, sodium-glucose cotransporter; RGR, renal glucose reabsorption. *Plasma glucose levels (<350 mg/dl) at which the maximum percentage inhibition of RGR was calculated.

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of the actual plasma tofogliflozin concentrations measured at three sampling points (60, 135, and 210 min after the start of infusion) were 4.6–474 ng/ml, which were 114 to 119% of the target plasma concentrations. Similarly, the means of the actual plasma phlorizin concentrations were 42.6 to 1,574 ng/ml, which were 106 to 118% of the target plasma concentrations.

The plasma glucose concentrations gradually increased from the normal range before glucose titration to over 400 mg/dl after 30 min infusion of 50% glucose solution (Fig. 1, A and B). During the experiments, the creatinine clearance of each group was stable at around 3 ml/min (Fig. 1, C and D).

Figure 2 shows the relationship between the plasma glucose concentration and UGE. In both the phlorizin group and the tofogliflozin group, the plasma glucose concentration at which apparent UGE was induced shifted to the left in a dose-dependent manner.

There were no significant differences between the glucose levels at which the maximum percentage inhibition of RGR was calculated (Table 1). The percentage inhibition of RGR increased dose-dependently in both the tofogliflozin group and the phlorizin group (Fig. 3). Over 50% inhibition of RGR was observed at ≥133 ng/ml of tofogliflozin (Fig. 3A) and at ≥400 ng/ml of phlorizin (Fig. 3B). Therefore, we next examined the effects of the SGLT inhibitors on UGE under hypo- and euglycemic conditions at 133 and 400 ng/ml for tofogliflozin and 400 and 1,333 ng/ml for phlorizin.
rizin; at these concentrations, the rSGLT2 will be completely inhibited according to their IC_{50} and protein-binding properties (21, 26).

**UGE under Hypo- and Euglycemic Conditions Induced by Glucose Clamp**

The means of actual plasma tofogliflozin concentrations at three sampling points (30, 90, and 150 min after infusion) in the 133 and 400 ng/ml groups were 245 and 599 ng/ml, which were 184 and 150% of the target plasma concentration, respectively. The means of actual plasma phlorizin concentrations at the three sampling points in the 400 and 1,333 ng/ml groups were 378 and 1,143 ng/ml, which were 95 and 86% of the target plasma concentration, respectively (Table 2).

By the continuous infusion of insulin for 30 min, the plasma glucose concentrations decreased from the normal range to nearly 50 mg/dl. Even with additional infusion of tofogliflozin or phlorizin for 60 min, plasma glucose concentrations remained above 40 mg/dl. Thereafter, with the glucose infusion at around 10–25 mg·kg^{-1}·min^{-1} for 30 min and around 25–35 mg·kg^{-1}·min^{-1} for 60 min, the plasma glucose concentration increased to the normal range and remained around 100 mg/dl for 60 min (Fig. 4).

We defined the UGE under hypoglycemic conditions as that during the last 30 min of the insulin plus tofogliflozin or insulin plus phlorizin infusion period, and the UGE under euglycemic condition as that during the last 30 min of insulin plus tofogliflozin plus glucose or insulin plus phlorizin plus glucose infusion as indicated in Fig. 4. There were no differences in plasma glucose concentration (Table 3) or in creatinine clearance (Table 4) between the five groups under either the hypoglycemic conditions.

Under hypoglycemic conditions, significant increases in UGE were observed in the phlorizin groups both at 400 and 1,333 ng/ml. In contrast, no significant increase in UGE was observed in tofogliflozin at 133 and 400 ng/ml under hypoglycemic conditions. Under euglycemic conditions, significant increases in UGE were also observed in the phlorizin groups both at 400 and 1,333 ng/ml. In contrast, no significant increase in UGE was observed in tofogliflozin at 133 ng/ml under euglycemic conditions, although a slight increase in UGE was induced with tofogliflozin at 400 ng/ml (Fig. 5).

**Effect of Acute UGE Induced by Tofogliflozin or Phlorizin on Plasma Glucose Levels and EGP**

Finally, we evaluated the effects on plasma glucose of continuous infusion of tofogliflozin (400 ng/ml) and phlorizin (1,333 ng/ml) under euglycemic conditions by simultaneously comparing UGE and EGP. The actual plasma tofogliflozin and phlorizin concentrations after 120 min infusion were 574 ± 80 and 1,514 ± 243 ng/ml, respectively.

Although tofogliflozin (400 ng/ml) induced UGE of about 2 mg·kg^{-1}·min^{-1} (Fig. 7A) and slightly decreased plasma glucose levels, the plasma glucose levels were maintained above 100 mg/dl (Fig. 7C).

### Table 2. Plasma concentration of SGLT inhibitors in the glucose clamp protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Plasma Concentration, ng/ml</th>
<th>n</th>
<th>30</th>
<th>90</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tofogliflozin</td>
<td>133</td>
<td>4</td>
<td>260.3 ± 24.0</td>
<td>193.8 ± 19.8</td>
<td>181.5 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>4</td>
<td>718.5 ± 27.8</td>
<td>556.5 ± 48.5</td>
<td>521.5 ± 48.5</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>400</td>
<td>4</td>
<td>364.8 ± 36.1</td>
<td>381.5 ± 18.3</td>
<td>387.0 ± 42.1</td>
</tr>
<tr>
<td></td>
<td>1,333</td>
<td>4</td>
<td>1,052.0 ± 67.3</td>
<td>1,177.5 ± 86.6</td>
<td>1,200.0 ± 87.6</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats.
with both the vehicle and tofogliflozin groups (Fig. 7A and Table 5). The minimum plasma glucose concentration during the steady-state period 60–120 min after the start of phlorizin infusion was significantly lower than that in the tofogliflozin group (Table 5). The calculated EGP in the steady-state period (60–120 min) in the phlorizin group was increased by about 4 mg·kg⁻¹·min⁻¹, which was greater than that in the tofogliflozin group (Fig. 7B and Table 5). However, there was a significant reduction in the Δ(EGP – UGE) value in the phlorizin group compared with that in the vehicle group and in the tofogliflozin group (Table 5), suggesting that the UGE induced with phlorizin may not be fully compensated for by the increase in EGP.

**DISCUSSION**

Although it had been generally believed that SGLT2 mediated 90% of RGR in humans (3, 25), recent clinical studies with SGLT2 inhibitors have shown only about 30–50% inhibition of RGR, provoking debate on the mechanisms underlying this discrepancy (9, 16, 23).

Liu et al. (16) proposed several explanations for the discrepancy and denied the possibility that SGLTs/glucose transporters other than SGLT2 were responsible for a much greater fraction of the RGR than previously reported. However, the electrophysiological studies and the titration study with SGLT2 knockout mice suggest the possibility of the increased contribution of SGLT1 under hypoglycemic conditions (9) or euglycemic conditions, especially with SGLT2 deficiency (24).

### Table 3. Plasma glucose levels under basal, hypoglycemic, and euglycemic conditions in the glucose clamp protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Plasma Concentration, ng/ml</th>
<th>n</th>
<th>Plasma Glucose, mg/dl</th>
<th>Basal</th>
<th>Hypoglycemia*</th>
<th>Euglycemia**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4</td>
<td></td>
<td>134 ± 7</td>
<td>46 ± 7</td>
<td>99 ± 3</td>
<td></td>
</tr>
<tr>
<td>Tofogliflozin</td>
<td>133</td>
<td>4</td>
<td>130 ± 9</td>
<td>48 ± 7</td>
<td>95 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>4</td>
<td>128 ± 4</td>
<td>40 ± 9</td>
<td>96 ± 3</td>
<td></td>
</tr>
<tr>
<td>Phlorizin</td>
<td>400</td>
<td>4</td>
<td>131 ± 11</td>
<td>43 ± 2</td>
<td>97 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,333</td>
<td>4</td>
<td>131 ± 4</td>
<td>46 ± 5</td>
<td>97 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats. *Mean values during the last 30 min of the insulin plus vehicle plus tofogliflozin or phlorizin infusion period, as indicated in Fig. 4. **Mean values during the last 30 min of insulin plus vehicle plus tofogliflozin or phlorizin plus glucose infusion period, as indicated in Fig. 4.

### Table 4. Creatinine clearance under basal, hypoglycemic, and euglycemic conditions in the glucose clamp protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Plasma Concentration, ng/ml</th>
<th>n</th>
<th>Creatinine Clearance, ml/min</th>
<th>Basal</th>
<th>Hypoglycemia*</th>
<th>Euglycemia**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4</td>
<td></td>
<td>2.33 ± 0.40</td>
<td>2.55 ± 1.22</td>
<td>3.34 ± 1.08</td>
<td></td>
</tr>
<tr>
<td>Tofogliflozin</td>
<td>133</td>
<td>4</td>
<td>2.67 ± 0.41</td>
<td>2.81 ± 0.35</td>
<td>2.52 ± 0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>4</td>
<td>1.95 ± 0.32</td>
<td>2.76 ± 0.74</td>
<td>3.11 ± 0.50</td>
<td></td>
</tr>
<tr>
<td>Phlorizin</td>
<td>400</td>
<td>4</td>
<td>2.81 ± 0.47</td>
<td>2.62 ± 0.76</td>
<td>2.88 ± 0.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,333</td>
<td>4</td>
<td>2.64 ± 0.44</td>
<td>3.01 ± 0.43</td>
<td>2.94 ± 0.77</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rats. *Mean values during the last 30 min of the insulin plus vehicle plus tofogliflozin or phlorizin infusion period, as indicated in Fig. 4. **Mean values during the last 30 min of insulin plus vehicle plus tofogliflozin or phlorizin plus glucose infusion period, as indicated in Fig. 4.
this is the case, inhibition of SGLT1 with SGLT2 inhibitors under hypoglycemic conditions or normalized glycemic conditions may have a significant impact on the physiological conditions of type 2 diabetes patients. Therefore, it is important to confirm the capacity of SGLT1 in RGR under hypoglycemic conditions and to know the relationship between the ratio of contribution of SGLT1 vs. SGLT2 and the glucose levels in vivo.

Although it is a feasible approach to use SGLT inhibitors to understand the roles of SGLT subtypes in RGR in vivo, there are limitations to the understanding that can be gained from comparisons of data from separate clinical studies of SGLT inhibitors. Intravenous infusion of phlorizin to humans was reported for the first time in 1933 by Chasis et al. (4). They reported that “tubular reabsorption of glucose was completely blocked” by phlorizin (11.8–65.2 mg/kg iv). Hummel et al. (9) proposed the greater (>50%) contribution of SGLT1 to tubular glucose reabsorption under normal conditions by comparing these results for phlorizin and the estimated percentage inhibition (about 50%) of glucose reabsorption in the clinical studies with dapagliflozin (13, 14). However, it is difficult to estimate the contribution of SGLT1 to tubular glucose reabsorption from these results for the following reasons. First, since the plasma phlorizin concentrations were not determined in the intravenous infusion studies with phlorizin in humans (4), it is impossible to examine the precise relationships between the inhibition of SGLT1/2 and UGE. In addition, although the plasma concentrations of dapagliflozin were determined in the clinical studies (13, 14), it is possible that fluctuations of plasma drug concentration caused by oral administration may impose limitations on the interpretation of the relationship between the drug concentrations in plasma and the efficacy. Moreover, the SGLT1 and SGLT2 contributions under different glycemic levels remain unknown.

In this study, we examined the contributions of SGLT2 and SGLT1 under different glycemic conditions by comparing the inhibitory effects of tofogliflozin, a highly specific SGLT2 inhibitor, and phlorizin, an SGLT1/2 inhibitor, on RGR with glucose titration and clamp protocols in normal rats. In particular, we conducted these experiments under fixed plasma concentrations of each SGLT inhibitor to evaluate the relationship between the inhibitory activities estimated from the plasma concentration and the inhibition of RGR.

Under hyperglycemic conditions (protocol 1), over 50% inhibition of RGR was achieved by tofogliflozin (≥133 ng/ml) and phlorizin (≥400 ng/ml) (Fig. 3). Based on the actual plasma concentrations (Table 1) and the protein-binding properties of tofogliflozin (21), we estimated the unbound tofogliflozin concentrations at 133 ng/ml (actual mean concentration: 168 ng/ml) and 400 ng/ml (actual mean concentration: 474 ng/ml) to be 70 and 196 nM, respectively. Considering the IC50 values of tofogliflozin against rSGLT1 and rSGLT2 (rSGLT1, 8,200 nM; rSGLT2, 15 nM) calculated from its inhibitory activities on the sodium-dependent uptake of α-methyl-D-glucopyranoside (AMG), a non-metabolizable glucose analog, in COS-7 cells overexpressing rSGLT1 or rSGLT2 (21), the unbound concentrations of tofogliflozin mentioned above are relevant concentrations to inhibit rSGLT2 almost completely but not rSGLT1.

Similarly, based on the actual plasma concentrations (Table 1) and the reported protein-binding properties of phlorizin (26), we estimated the unbound phlorizin concentrations at 400 ng/ml (actual concentration: 433 ng/ml) and 1,333 ng/ml (actual concentration: 1,574 ng/ml) to be 309 and 1,123 nM, respectively. Considering the IC50 values of phlorizin against rSGLT2 in our AMG uptake assay (48 nM) (21), and the reported IC50 values against rSGLT2 of phlorizin (8, 22), the unbound phlorizin concentrations mentioned above are also relevant concentrations to inhibit rSGLT2 almost completely.

Because the inhibition of rSGLT1 at the estimated unbound phlorizin concentration (309 and 1,123 nM) in our AMG uptake assay (21) was estimated to be about 30–50%, the inhibitory activity of phlorizin on RGR in our experiment is expected to be mainly due to the partial inhibition of rSGLT1 and complete inhibition of rSGLT2. Recently, SGLT3 has been
identified in the human kidney as a novel sodium transporter sensitive to phlorizin (15). Although mRNA for SGLT3b, an ortholog of hSGLT3, has been found in rat kidney (GenBank DQ054787), its function in rat kidney is still unknown. Even if the SGLT3b functions as a glucose transporter in rat kidney, as the sugar transport activity of mouse SGLT3b is estimated to be 60 times lower than that of mouse SGLT1 (1) and the inhibitory activity of phlorizin against hSGLT3 is about 100 times lower than that against hSGLT1 in our sodium-dependent AMG uptake assay (21), the inhibitory activity of phlorizin against the glucose transport of rat kidney via SGLT3b may have a negligible impact on the results of this study.

The inhibitory effect of tofogliflozin on RGR was saturated at about 60% at 133–400 ng/ml under hyperglycemic conditions (Fig. 3A), where rSGLT2 was expected to be inhibited almost completely but not rSGLT1. In contrast, no saturation was observed in the inhibitory effect on RGR by phlorizin at 400–1,333 ng/ml (Fig. 3B), resulting in greater RGR inhibition at 1,333 ng/ml phlorizin than at 400 ng/ml tofogliflozin (73 ± 5%, phlorizin 1,333 ng/ml, 61 ± 5%, tofogliflozin 400 ng/ml; \( P < 0.05 \)). At 1,333 ng/ml phlorizin, rSGLT2 was expected to be inhibited almost completely with a substantial rSGLT1 inhibition by about 50%. Therefore, the difference in RGR inhibition (%) between phlorizin (1,333 ng/ml) and tofogliflozin (400 ng/ml) was attributed to the partial inhibition of rSGLT1 by phlorizin. Taken together, the contribution of rSGLT2 to the RGR under hyperglycemic conditions was assumed to be about 60% in rats.

Under hypo- and euglycemic conditions with glucose clamp (protocol 2), phlorizin reduced RGR by about 25–35% and 50–60% at 400 and 1,333 ng/ml, respectively, where rSGLT2 is almost totally inhibited and rSGLT1 is partially inhibited. In contrast, tofogliflozin minimally (1–5%) reduced RGR under hypoglycemic conditions even at the concentrations supposed to inhibit rSGLT2 almost completely (Fig. 6). Because the actual concentrations of phlorizin and tofogliflozin were maintained at the same levels (Table 2) and the plasma glucose levels and creatinine clearance were stable during the measurement of RGR inhibition (Tables 3 and 4), the minimal inhibition of RGR with tofogliflozin is the result of the absence of rSGLT1 inhibition. In SGLT2 knockout mice, a greater contribution of SGLT1 to RGR under euglycemic conditions has been proposed (23, 24). Our results not only strongly support these suggestions but also suggest the dominant role of SGLT1 under euglycemic conditions.

**Table 5. Plasma glucose, EGP, and UGE at steady state in infusion protocol**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 9)</th>
<th>Tofogliflozin (n = 9)</th>
<th>Phlorizin (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>119 ± 11</td>
<td>109 ± 7*</td>
<td>102 ± 9***</td>
</tr>
<tr>
<td>Minimum plasma glucose, mg/dl†</td>
<td>117 ± 11</td>
<td>106 ± 7</td>
<td>96 ± 9***#</td>
</tr>
<tr>
<td>EGP, mg·kg⁻¹·min⁻¹</td>
<td>7.3 ± 1.0</td>
<td>8.8 ± 1.5*</td>
<td>11.0 ± 1.3***##</td>
</tr>
<tr>
<td>UGE, mg·kg⁻¹·min⁻¹</td>
<td>0.0071 ± 0.0019</td>
<td>2.3 ± 0.7***</td>
<td>5.7 ± 0.7***###</td>
</tr>
<tr>
<td>∆EGP-UGE, mg·kg⁻¹·min⁻¹</td>
<td>7.3 ± 1.0</td>
<td>6.5 ± 1.1</td>
<td>5.3 ± 1.0***##</td>
</tr>
</tbody>
</table>

Values are means ± SD during the last 60 min of vehicle, tofogliflozin, or phlorizin infusion period; \( n \), no. of rats. EGP, endogenous glucose production; UGE, urinary glucose excretion. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) vs. vehicle group by Dunnett’s multiple-comparison test. #\( P < 0.05 \), ##\( P < 0.01 \), and ###\( P < 0.001 \) vs. tofogliflozin group by unpaired t-test. †Mean values ± SD of the minimum value of three sampling times (60, 90, and 120 min after infusion) for each rat.
in RGR under hypoglycemic conditions. To evaluate whether this dominant role of SGLT1 is observed only under complete SGLT2 inhibition or not, it will be necessary to measure the actual glucose concentration gradient along the different segments of the proximal tubules.

Finally, we compared the UGE and EGP simultaneously to evaluate the hypoglycemic potentials of the SGLT inhibitors. Under euglycemic conditions, tofogliflozin-induced UGE and EGP were increased together with a slight decrease in plasma glucose concentration. Even after 120 min of tofogliflozin infusion at 400 ng/ml, the plasma glucose levels were maintained above 100 mg/dl. The increased EGP (1–2 mg·kg⁻¹·min⁻¹) was nearly the same as the UGE level induced with tofogliflozin. These results suggest that UGE induction with tofogliflozin under euglycemic conditions can be fully compensated for by the increase of EGP.

In contrast, compared with tofogliflozin, chlorphenirin inhibited greater UGE under euglycemic conditions, which may be the result of the dual inhibition of both SGLT1 and SGLT2. In the chlorphenirin group, although the EGP was also increased (by about 4 mg·kg⁻¹·min⁻¹, which was greater than in the tofogliflozin group), the plasma glucose decreased more than in the tofogliflozin group. Because the level of UGE induced with chlorphenirin (about 6 mg·kg⁻¹·min⁻¹) was apparently greater than the increased level of EGP, it is suggested that the induction of UGE with the dual inhibition of both SGLT1 and SGLT2 was not fully compensated for by the increase in EGP. The actual blood glucose-lowering effects have not been mentioned in studies in rats (19) or humans (4) treated with chlorphenirin under euglycemic conditions. Even in our experiment, actual hypoglycemia was not observed with continuous infusion of chlorphenirin for 120 min. However, the level of UGE observed with chlorphenirin, which was comparable to about 75% of the basal EGP (Fig. 7), suggests that dual inhibition of both SGLT1 and SGLT2 may pose a risk of excessive UGE under hypo- and euglycemic conditions, which may lead to sustained hypoglycemia. Further studies are required to understand the mechanisms of compensatory EGP increase and the long-term effects of sustained UGE induction by SGLT inhibitors.

In this study we examined the potential risk of hypoglycemia resulting from SGLT1 inhibition accompanying SGLT2 inhibition in normal rats. Although our results suggest the better profile of highly specific SGLT2 inhibition, experiments under diabetic conditions will be needed to precisely examine the potential risk of these compounds. Moreover, the mechanism that regulates the differential contributions of SGLT1 and SGLT2 to RGR under different glycemic conditions will need to be clarified.

In conclusion, the contribution of SGLT1 to RGR was found to be greater under lower glycemic conditions than under hyperglycemic conditions, and selective SGLT2 inhibition by tofogliflozin exhibited greater reduction of RGR preferentially under hyperglycemic conditions. This suggests that SGLT2-selective inhibitors, such as tofogliflozin, carry a lower risk of causing hypoglycemia than SGLT1/2 inhibitors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


