SGLT2 inhibitor lowers serum uric acid through alteration of uric acid transport activity in renal tubule by increased glycosuria

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ABSTRACT: Sodium glucose cotransporter 2 (SGLT2) inhibitors have been reported to lower the serum uric acid (SUA) level. To elucidate the mechanism responsible for this reduction, SUA and the urinary excretion rate of uric acid (UE_{UA}) were analysed after the oral administration of luseogliflozin, a SGLT2 inhibitor, to healthy subjects. After dosing, SUA decreased, and a negative correlation was observed between the SUA level and the UE_{UA}, suggesting that SUA decreased as a result of the increase in the UE_{UA}. The increase in UE_{UA} was correlated with an increase in urinary D-glucose excretion, but not with the plasma luseogliflozin concentration. Additionally, *in vitro* transport experiments showed that luseogliflozin had no direct effect on the transporters involved in renal UA reabsorption. To explain that the increase in UE_{UA} is likely due to glycosuria, the study focused on the facilitative glucose transporter 9 isoform 2 (GLUT9ΔN, *SLC2A9b*), which is expressed at the apical membrane of the kidney tubular cells and transports both UA and D-glucose. It was observed that the efflux of [^{14}C]UA in *Xenopus* oocytes expressing the GLUT9 isoform 2 was *trans*-stimulated by 10 mM D-glucose, a high concentration of glucose that existed under SGLT2 inhibition. On the other hand, the uptake of [^{14}C]UA by oocytes was *cis*-inhibited by 100 mM D-glucose, a concentration assumed to exist in collecting ducts. In conclusion, it was demonstrated that the UE_{UA} could potentially be increased by luseogliflozin-induced glycosuria, with alterations of UA transport activity because of urinary glucose. © 2014 The Authors. *Biopharmaceutics & Drug Disposition*. Published by John Wiley & Sons Ltd.

Key words: sodium glucose cotransporter; SGLT2 inhibitor; GLUT9; uric acid; urinary glucose excretion

Introduction

The sodium glucose cotransporter 2 (SGLT2) is expressed predominantly in the S1 segment of the proximal tubule in kidney and accounts for 90% of the renal reabsorption of glucose [1,2].

The SGLT2 inhibitors are a new class of antidiabetic medications that decrease plasma glucose levels by blocking SGLT2-mediated glucose reabsorption [3,4]. Late phase clinical trials with SGLT2 inhibitors have shown the efficacy and safety of these inhibitors in patients with type 2 diabetes. In addition, decreased serum uric acid (SUA) levels have been observed [5–8] for all the SGLT2 inhibitors including luseogliflozin [9,10]. About four decades ago, a non-selective SGLT inhibitor, phloridzin, was reported to exert a uricosuric effect as a result of an osmotic effect

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and possibly to modulate uric acid (UA) transport in healthy subjects [11]. However, the exact mechanism of the uricosuric effect of phloridzin remains unclear.

Elevated serum uric acid levels are associated with gout attacks [12,13]. In addition, recent evidence suggests a significant association between hyperuricemia and chronic kidney disease [14,15], metabolic syndrome [16,17], hypertension [18,19] or cardiovascular events [20,21]. The serum uric acid levels depend on a balance between the production and excretion of uric acid, in which approximately 70% of the uric acid is excreted into the urine and the remainder is thought to be excreted into the intestinal tract. The renal transport system plays an important role in the regulation of the SUA level and involves reabsorption and secretion. Consequently, ~90% of glomerularly filtered uric acid is reabsorbed by the epithelial cells of the proximal tubules [22,23]. Recently, transporters involved in the renal handling of uric acid have been identified. Uric acid is reabsorbed by the proximal tubule cells mainly via the uric acid transporter 1 (URAT1, SLC22A12) [24,25], possibly via organic anion transporter 4 (OAT4, SLC22A11) [26,27] and OAT10 (SLC22A13) [27], and is transported to the blood via GLUT9 isoform 1 (SLC2A9a) [25,28–31]. Furthermore, uricosuric agents (such as benzbromarone and probenecid), angiotensin II receptor blockers (such as losartan) and salicylic acid inhibit URAT1, and this explains the SUA-lowering effects of these drugs [24,32]. To date, the effects of SGLT inhibitors on transporters involved in the renal handling of uric acid are unknown.

Hyperuricemia is closely linked to diabetes, since insulin resistance is correlated with SUA levels but inversely correlated with the renal clearance of curic acid (CL_{UA}) [33,34]. Insulin has been suggested to increase uric acid reabsorption in the proximal tubule [34]. On the other hand, when the disease progresses to the stage of glycosuria, the serum uric acid level begins to decrease [35–38]. These mechanisms have been proposed as an effect of glucose on uric acid handling in the proximal tubule [35,37]. This phenomenon is similar to the SUA-lowering effect of SGLT2 inhibitors.

The present study describes the SUA-lowering effect of luseogliflozin in clinical studies to assess the safety, pharmacokinetics and glucose-excreting effect of luseogliflozin in healthy subjects [39]. In addition, in vitro experiments were performed using Xenopus oocytes and cultured cells expressing uric acid transporters to elucidate the mechanism.

Materials and Methods

Subjects and study design

Fifty-seven and 24 healthy Japanese men participated in a single dose study and a multiple dose study of luseogliflozin, respectively (Table 1). The subjects were between the ages of 20 and 39 years and were in good health as assessed by screening examinations, a complete medical history and a physical examination. Subjects were excluded from the studies if they had any clinically significant disease or had experienced a significant body weight change (±3 kg) within 4 weeks of the first administration or the use of any drugs within the first week of the first administration. All subjects provided written informed consent prior to their participation in the studies. For the single dose study, 57 eligible individuals were randomly assigned to the following treatment groups: placebo, 1, 3, 5, 9, 15 or 25 mg luseogliflozin. After fasting for at least 10 h, luseogliflozin was administered orally. For the multiple dose study, 24 eligible individuals were randomly assigned to the following treatment groups: placebo, 5 or 10 mg. Luseogliflozin was administered orally before breakfast once daily for 7 days. Blood and urine samples were collected at the predetermined time points. The studies complied with the Helsinki

Table 1. Demographic and baseline characteristics

Clinical characteristic	Single dose study	Multiple dose study
Subjects (males)	57	24
Age (years)	26.5 ± 5.5	28.9 ± 5.9
Body weight (kg)	61.8 ± 5.9	62.4 ± 6.8
HbA1c (%)	5.1 ± 0.2	5.1 ± 0.2
HbA1c (mmol/mol)	32 ± 3	32 ± 3
FPG (mg/dl)	91.1 ± 4.8	89.3 ± 5.7
UE_{GL} (g/day)	0.147 ± 0.206	0.153 ± 0.169
SUA (mg/dl)	5.7 ± 0.8	5.5 ± 0.9
UE_{UA} (mg/day)	556 ± 90	589 ± 91
CL_{UA} (ml/min)	6.88 ± 1.29	7.68 ± 2.20

FPG, fasting plasma glucose; UE_{GL}, urinary excretion rate of glucose; SUA, serum uric acid; UE_{UA}, urinary excretion rate of uric acid; CL_{UA}, renal clearance of uric acid. Data are mean ± SD.

Declaration, the standards of the Japanese Pharmaceutical Affairs Law, and the Good Clinical Practice guidelines. The study protocols were approved by an Institutional Review Board (Kyushu Clinical Pharmacology Research Clinic, Fukuoka, Japan). The studies were registered with the Japan Pharmaceutical Information Center (JapicCTI-132353, JapicCTI-132354) [39].

Measurements

Blood was centrifuged (4 °C, 3000 rpm, 15 min) immediately after collection to obtain plasma samples. The concentrations of glucose and uric acid in the plasma and urine were measured using an automatic analyser (TBA-120FR, Toshiba Medical Systems Corp. or Glucoroder-NX, A&T Corp.). Concentrations of luseogliflozin in the plasma and urine were determined using HPLC tandem mass spectrometry (API4000; Applied Biosystems/MDS Sciex, Foster City, CA, USA) after pre-treatment with solid phase extraction (OASIS HLB, Waters Corp., Milford, MA, USA). The lower limit of quantification was 0.05 ng/ml for plasma and 0.5 ng/ml for urine, respectively. The CL_{UA} were calculated from the plasma concentration and urinary excretion data. The AUC_{0-24h} of the plasma luseogliflozin concentrations was calculated using a standard noncompartmental method.

Transporter inhibition experiments in oocytes

The preparation of *Xenopus laevis* oocytes, the *in vitro* synthesis of cRNA (GLUT9 isoform 1, OAT10 or SMCT1) and the uptake experiments using [14C]UA (20 μM) (except for SMCT1: [3H] nicotinic acid, 15 μM) were conducted as described previously [40–43]. For the inhibition experiment, uptake was initiated by replacement with a transport buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$ and 5 mM HEPES, pH 7.4) containing [14C]UA or [3H]nicotinic acid and luseogliflozin (Taisho Pharmaceutical, Saitama, Japan); for each condition, 8–10 oocytes prepared from a single batch, were incubated for 60 min at 25 °C. The uptake reaction was terminated by washing the oocytes with ice-cold transport buffer, and the oocytes were then solubilized in 5% SDS for the quantification of radioactivity. The uptake rate (μl/min/oocyte) was calculated by dividing the uptake amount by the initial concentration of the substrate in the

transport buffer. The transporter-mediated uptake rate was obtained after subtracting the uptake of the water-injected oocytes from that of the cRNA-injected oocytes. The inhibitory effect of luseogliflozin was expressed as the percentage of the control.

Transporter inhibition experiments in cultured cells

The inhibition experiments were performed using stably URAT1-expressing HEK293 cells and OAT4-expressing S2 cells (established from S2 segments of mouse renal proximal tubules) in a contracted laboratory in a manner similar to that described previously [40]. The URAT1-expressing HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin, streptomycin, amphotericin B and 2 mM L-glutamine. The OAT4-expressing S2 cells were cultured in RITC80-7 medium containing 5% FBS, 10 μg/l EGF, 0.08 units/ml insulin and 10 mg/l transferrin, in a humidified 5% CO$_2$ and 95% air atmosphere at 37 °C. For the inhibition experiment, three wells of cells for each condition were preincubated in transport buffer (Hanks' balanced salt solution) containing luseogliflozin for 15 min at 37 °C. Uptake was initiated by replacement with transport buffer containing [14C]UA (50 μM) and the cells were incubated for 2 min (URAT1) or 15 min (OAT4) at 37 °C. The cells were washed three times with ice-cold PBS and solubilized in 0.1 N NaOH for the quantification of radioactivity. Part of the lysate was subjected to the protein measurement. The uptake rate (μl/min/mg protein) was calculated by dividing the uptake amount by the initial concentration of the substrate in the transport buffer and the protein amount of the cells. The transporter mediated uptake rate and the inhibitory effect were calculated in the same manner as that of the oocytes from the values of the control cells (transfected with the vector only) and the cRNA-transfected cells.

Effects of D-glucose on UA transport mediated by GLUT9 isoform 2

For the *trans*-stimulatory experiment on GLUT9 isoform 2-mediated UA efflux, *Xenopus laevis* oocytes were injected with 0.1−25 ng of cRNA of GLUT9 isoform 2 (Genbank NM_001001290.1) or water and cultured at 18 °C for 1 day. The UA-efflux

experiments were performed at 25 °C using 8–10 oocytes prepared from a single batch for each group. Immediately after the injection of 50 nl of [^{14}C]UA solution (1 mM) into the oocyte, the efflux reaction was individually initiated by replacement with 100 μl of transport buffer. After the reaction had been allowed to occur for the designated time, transport buffer and oocyte were recovered. The radioactivity in buffer and oocytes was quantified to calculate the efflux ratio of [^{14}C]UA as the percentage of the amount remaining within the oocytes or released into the buffer relative to the to-tal radioactivity. The transporter-mediated efflux rate was calculated by subtracting the values of the water-injected oocytes from those of the GLUT9 isoform 2-expressing oocytes. To investigate the effect on the UA efflux, D-glucose was dissolved in the transport buffer at concentrations of 5 or 10 mM. The efflux reaction was performed for 5 min. As a control study, transport buffer with 10 mM of L-glucose was used. Additionally, to investigate the effect on the GLUT9 isoform 2-mediated UA efflux, benzbromarone was dissolved in the trans-port buffer containing 10 mM D-glucose at a concen-tration of 100 μM. For the stimulatory experiment on GLUT9 isoform 2-mediated UA uptake, the oocytes were injected with 25 ng of cRNA of GLUT9 isoform 2 or water and cultured for 2 days. After the injection of 50 nl of D-glucose or L-glucose solution (100 mM each), the uptake of [^{14}C]UA (10 μM) was performed at 25 °C for 15 min using 18 oocytes for each group in transport buffer in which Na$^+$ had been replaced with K$^+$. The transporter-mediated uptake rate (μl/15 min/oocyte) was calculated in the same manner as described above. For the inhibition experiment on the UA uptake by D-glucose, uptake was performed using 10–12 oocytes for each group in transport buffer with 10 or 100 mM of D-glucose. As a control study, the ef-fect of 100 mM D-mannitol, 100 mM L-glucose and transport buffer without any sugar were investigated. The other conditions were the same as the experiment for the stimulatory effect of D-glucose on UA uptake.

Uric acid uptake by SGLT2

The uptake experiments using stably SGLT2 expressing CHO-K1 cells were performed as described previously [10]. After preincubation of the SGLT2-transfected cells and the control cells in Na$^+$-free buffer (140 mM choline chloride, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 5 mM Tris, pH 7.4) at 37 °C for 20 min, uptake was initiated by replacing Na$^+$-free buffer with trans-port buffer (140 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 5 mM Tris, pH 7.4) containing [^{14}C]UA (9 μM) or methyl-α-D-[U-^{14}C]-glucopyranoside ([^{14}C]α-MG, 1 mM). Uptake was allowed to occur for 5 min and was then terminated by washing with ice-cold transport buffer. The uptake rate (μl/5 min/mg protein) was calculated in the same manner as described above.

Statistical analyses

Data were expressed as the mean ± SEM or SD. Statistical significance was determined using Student's t-test or Aspin-Welch's t-test after an analysis of variance when comparing two groups and using Dunnett's test for multiple-group comparisons. A simple linear regression analysis was performed using the standard method. A value of $p < 0.05$ was considered significant.

Results

SUA-lowering effect and uricosuric effect of luseogliflozin

The laboratory parameters from the first two clin-ical studies of luseogliflozin in healthy subjects were analysed [39]. Changes in the serum uric acid levels from the baseline values are shown in Figure 1. In the single dose study, the SUA level was significantly decreased at doses higher than 1 mg vs the placebo on day 1, and changes from the baseline were −0.66 to −1.59 mg/dl (Figure 1A). In the multiple dose study, the SUA level was also significantly decreased at doses of 5 mg and 10 mg vs the placebo on day 1, and the changes were −1.39 and −1.33 mg/dl (Figure 1B). On day 3, the changes in the SUA level were further increased to −1.76 and −1.71 mg/dl, which were comparable to those observed on day 7.

Changes in the urinary excretion rate of uric acid (UE_{UA}) and the CL_{UA} from the baseline are shown in Figure 2. The UE_{UA} was significantly increased for all doses on day 1 vs the placebo in both the single dose and the multiple dose studies. Changes from the baseline were 189–268 mg/day
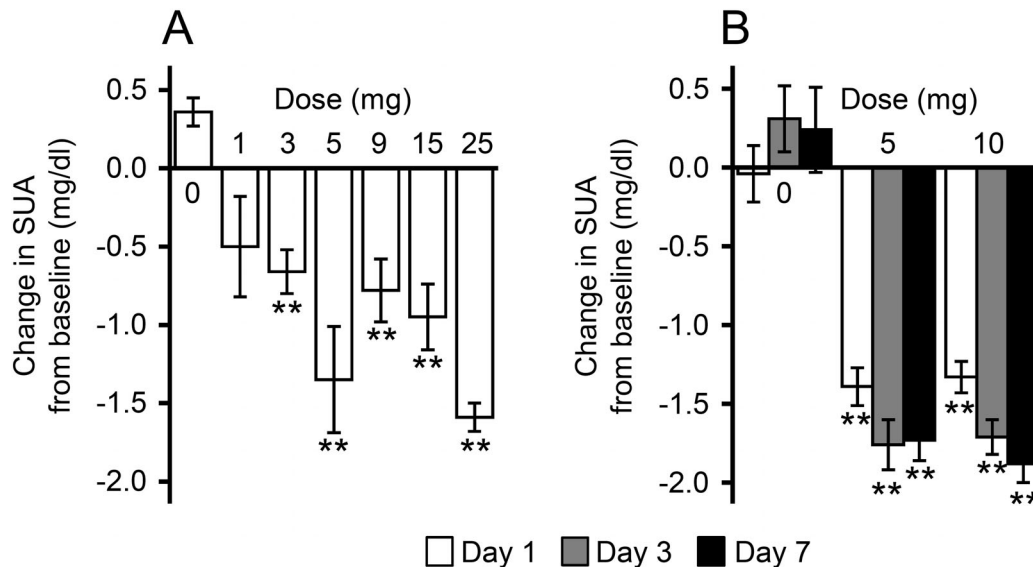
Figure 1. Effect of luseogliflozin on the serum uric acid (SUA) level. Changes in the SUA level from the baseline after a single dose (A, $n = 3–14$) and after multiple doses (B, $n = 8$) are shown. Data are mean ± SEM. **$p < 0.01$ vs placebo (0 mg) (Dunnett's test)

and 243–279 mg/day for the single dose and multiple dose studies, respectively, on day 1 (Figure 2A, B). In the multiple dose study, the change in the UE_{UA} from the baseline was the largest on day 1, with the magnitude of change decreasing on days 3 and 7 (Figure 2B). On the other hand, the CL_{UA} was increased on days 1–7 in both dose groups in the multiple dose study (Figure 2D).

Association of SUA with UE_{UA} after administration of luseogliflozin

Changes in the SUA and UE_{UA} levels from the baseline values in the three representative dose groups (1, 5 and 25 mg) are shown in Figure 3A, B, respectively. In the 1 mg dose group, the decrease in the SUA level was not significant on day 1 and the UE_{UA} was increased on day 1 but had returned to the baseline value on day 2. In the 25 mg group, a marked reduction in the SUA value continued until day 4, while the increase in the UE_{UA} value continued until at least day 2. In the 5 mg group, the changes in the serum uric acid and UE_{UA} levels were intermediate to those for the previous two doses. The correlation between the changes in the serum uric acid level and the UE_{UA} relative to the baseline on day 1 for all subjects (1–25 mg) is shown in Figure 3C. The change in the SUA level showed a strong negative correlation with the change in the UE_{UA} ($r = -0.7672$, $p < 0.001$).

Association of UE_{UA} with UE_{GL} or plasma concentration of luseogliflozin

The time courses of the increase in UE_{UA} vs the placebo group, the plasma concentration of luseogliflozin and the increase in the urinary excretion rate of glucose (UE_{GL}) vs the placebo group at the three representative doses are shown in Figure 4A–C. The increase in the UE_{UA} was relatively high at two time points (at 2–8 h and at 12–16 h post-dose, Figure 4A), whereas the plasma concentration of luseogliflozin reached a maximum at 1 h post-dose and then decreased (Figure 4B). The increase in the UE_{GL} showed a similar transition to that of the UE_{UA}, since the UE_{GL} increased markedly at around 5 h and 13 h post-dose after food intake (Figure 4C). Additionally, the correlation between the changes in UE_{UA} and UE_{GL} values was compared with the correlation between the changes in UE_{UA} and the plasma AUC values after a single dose of luseogliflozin (Figure 4D, E). The change in UE_{UA} was more strongly correlated with the change in UE_{GL} ($r = 0.7875$, $p < 0.001$, Figure 4D), than with the AUC ($r = 0.5707$, $p < 0.001$, Figure 4E).

Effect of luseogliflozin on transporters involved in renal uric acid reabsorption

To determine whether luseogliflozin has a direct uricosuric effect on uric acid handling in the kidney,
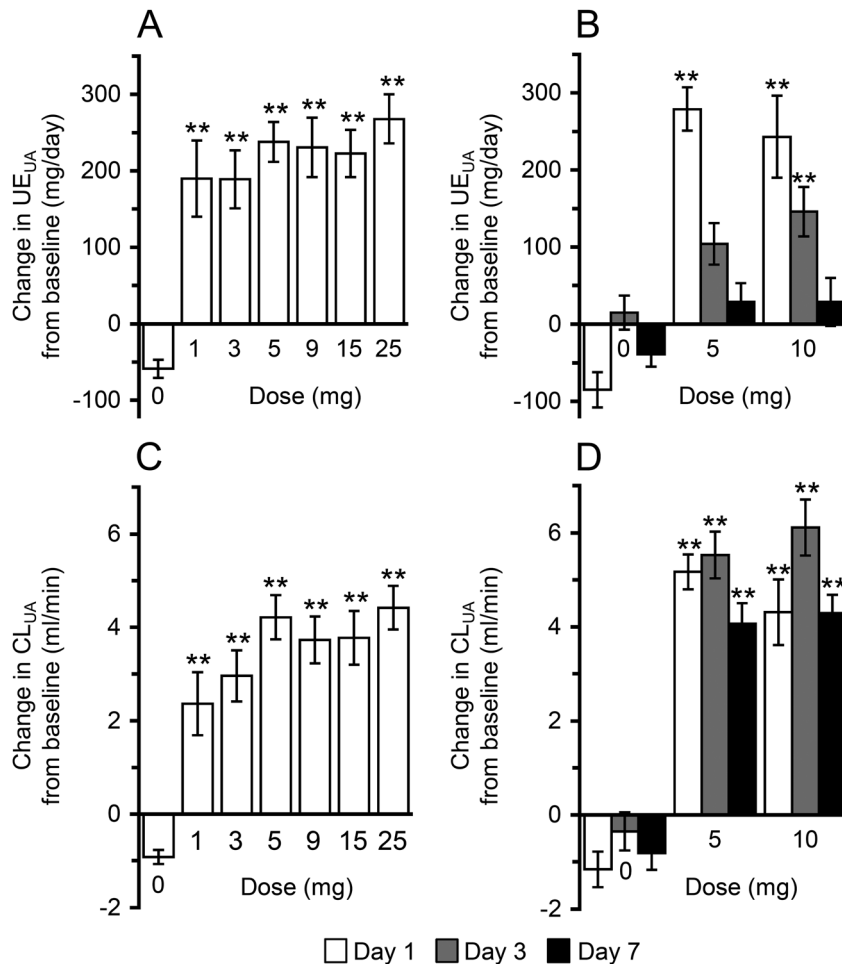
Figure 2. Effects of luseogliflozin on the urinary excretion rate (UE_{UA}) and the renal clearance (CL_{UA}) of uric acid. Changes in UE_{UA} and CL_{UA} from the baseline after a single dose (A and C, $n = 3$–14) and after multiple doses (B and D, $n = 8$) are shown. Data are mean ± SEM. **$p < 0.01$ vs placebo (0 mg) (Dunnett's test)

the effect of luseogliflozin on [14C]UA transport mediated by URAT1, GLUT9 isoform 1, OAT4 or OAT10 was examined using *in vitro* cell culture models expressing the respective transporter genes. Additionally, because SMCT1 transports monocarboxylic acids, such as lactic acid and nicotinic acid, which stimulate the exchange transport of uric acid via URAT1 [43,44], the effect of luseogliflozin on SMCT1-mediated [3H]nicotinic acid transport was also examined. Luseogliflozin was found not to affect the activities of any of the transporters (Figure 5).

Uric acid transport by SGLT2

The study examined uric acid transport in CHO-K1 cells stably expressing SGLT2 to test the hypothesis

that UE_{UA} should be increased after treatment with SGLT2 inhibitors. The uptake of a known substrate, [14C]α-MG, by SGLT2-expressing cells was significantly greater than that by control cells (8.15 ± 1.49 vs 2.35 ± 0.96 µl/5 min/mg protein, mean ± SEM). On the other hand, the uptake of [14C]UA by SGLT2-expressing cells and control cells were 1.73 ± 0.44 µl/5 min/mg protein and 2.12 ± 0.75 µl/5 min/mg protein (mean ± SEM), respectively, suggesting that uric acid was not transported by SGLT2.

Trans-stimulatory effect of D-glucose on uric acid transport by GLUT9 isoform 2

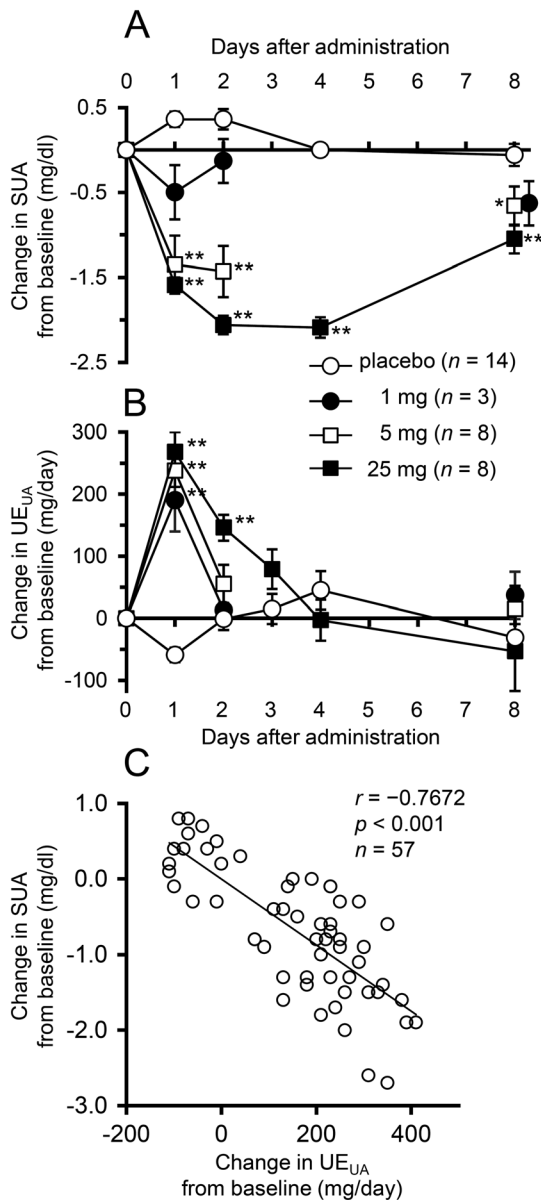The *trans*-stimulatory effect of D-glucose on efflux of [14C]UA mediated by GLUT9 isoform 2 was

Figure 3. Relationship between the serum uric acid (SUA) level and the urinary excretion rate of uric acid (UE_{UA}) after a single dose. The daily changes in the SUA level (A) and UE_{UA} (B) from the baseline in the 1, 5 and 25 mg dosing groups are shown. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs placebo (Dunnett's test, Student's t-test or Aspin-Welch's t-test). (C) Correlation between the changes in the SUA level and the UE_{UA} on day 1 from the baseline values in the single dose study

examined using oocytes expressing GLUT9 isoform 2. The [^{14}C]UA efflux rate was clearly changed depending on the amount of cRNA of GLUT9 isoform 2 injected into the oocytes at a range of 0.1–25 ng

(Figure 6A) and increased linearly over 15 min (Figure 6B). Based on these results, in order to more clearly evaluate [^{14}C]UA efflux, oocytes injected with a smaller amount (0.25 ng) of cRNA in a shorter measurement time (5 min) compared with the ordinary conditions were examined [31,41]. The efflux of [^{14}C]UA injected into oocytes expressing GLUT9 isoform 2 in the presence of 10 mM D-glucose was significantly higher than that of 10 mM L-glucose (Figure 6C). Additionally, the stimulated [^{14}C]UA efflux by 10 mM of D-glucose was decreased in the presence of 100 μM of benzbromarone [29–31] (Figure 6D). To confirm the exchange transport of UA with D-glucose, the *trans*-stimulatory effect of D-glucose on [^{14}C]UA uptake was examined upon injection of 50 nl of 100 mM of D-glucose solution into oocytes just prior to [^{14}C]UA uptake by the GLUT9 isoform 2-expressing oocytes; uptake was indeed significantly higher than that of 100 mM of L-glucose (Figure 6E).

Cis-inhibitory effect of D-glucose on uric acid uptake by GLUT9 isoform 2

The *cis*-inhibitory effect of D-glucose on GLUT9 isoform 2-mediated [^{14}C]UA uptake was examined at concentrations of 10 and 100 mM. A significant difference was observed between the groups of control (transport buffer) and 100 mM D-glucose ($p < 0.05$, Aspin-Welch's t-test), although no significant difference was observed as a result of multiple comparison (Dunnett's test) (Figure 7).

Discussion

The present study elucidated the mechanism of the SUA-lowering effect that is commonly observed in response to SGLT2 inhibitors [5–8] using luseogliflozin as a model drug.

First, the study evaluated whether the change in the serum uric acid level can be attributable to the change in the UE_{UA} using an SGLT2 inhibitor in clinical studies. When luseogliflozin was administered to healthy subjects, a decrease in the SUA level and an increase in the UE_{UA} were observed (Figures 1 and 2). After a single administration (1–25 mg), a negative correlation was observed between changes in the serum uric acid level and
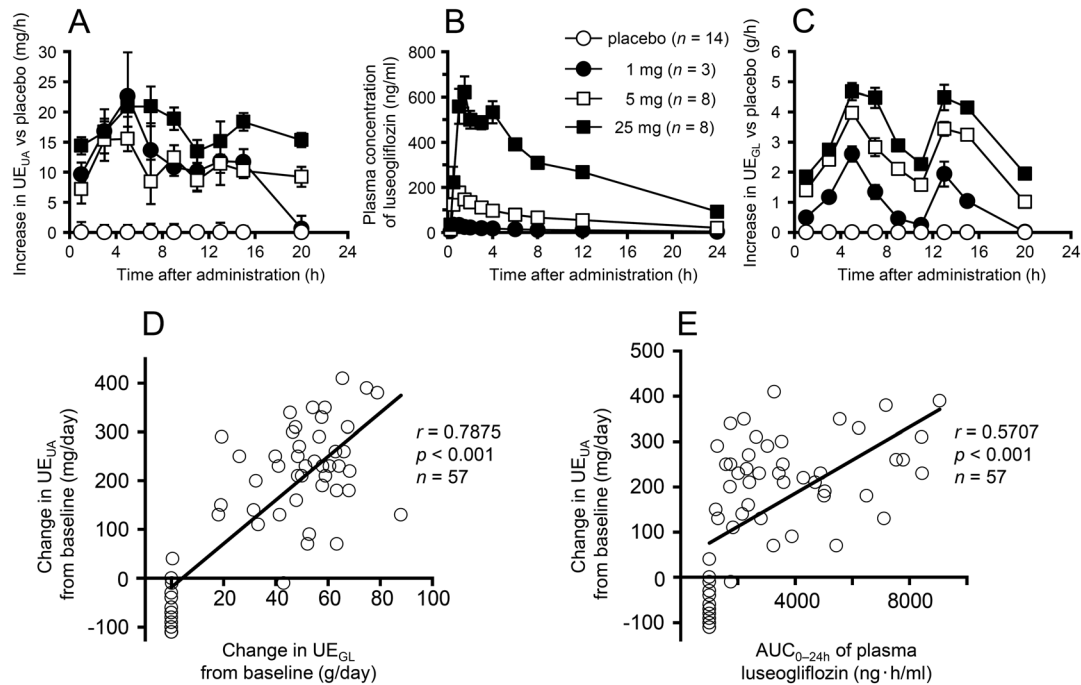
Figure 4. Comparison of the urinary excretion rate of uric acid (UE_{UA}), the plasma concentration of luseogliflozin and the urinary excretion rate of glucose (UE_{GL}) in the single dose study. (A–C) Time course profiles of UE_{UA}, the plasma concentration of luseogliflozin and UE_{GL}. Data are mean ± SEM. (D) Relationship between UE_{UA} and UE_{GL}. (E) Relationship between UE_{UA} and AUC_{0-24h} of plasma luseogliflozin

the UE_{UA} (Figure 3C). In addition, the increase in the UE_{UA} from the baseline value on day 1 accounted for 33%−46%. Although this percentage was greater than that of the decrease in the SUA level (8%−25%), the extent of the increase in the UE_{UA} was considered reasonable as an explanation for the decrease in the SUA level, since the total uric acid in the body is approximately 1200 mg in healthy subjects [45]. These observations suggest that the SUA-lowering effect is due to an increase in the UE_{UA}. In addition, the increase in the UE_{UA} after multiple dosing was marked on day 1 and decreased on days 3 and 7 (Figure 2B), probably because of the decrease in the SUA level. The CL_{UA} remained at a high level until day 7 (Figure 2D), suggesting that the SUA-lowering effect continued.

Second, the mechanism responsible for the uricosuric effect of an SGLT2 inhibitor was examined. Previously, Skeith et al. reported that a non-selective SGLT inhibitor, phloridzin, and D-glucose exerted a uricosuric effect that was stronger than that of mannitol after a bolus infusion in humans [11]. Hence, the effects of phloridzin and D-glucose

were estimated to be due not only to osmotic diuresis, but also to an effect on uric acid handling in the kidney. Additionally, Knight et al. reported that both phloridzin and D-glucose inhibited uric acid reabsorption in rat proximal tubules [46], while the mechanisms underlying their uricosuric effects were unclear. Based on our clinical studies and these previous observations, it was postulated that the glucose level is directly related to the SUA-lowering effect of SGLT2 inhibitors. When the dose of luseogliflozin was changed from 1 mg to 25 mg, the time profile for the increase in the UE_{UA} was comparable to that for the UE_{GL}, but not with that for the plasma concentration of luseogliflozin (Figure 4A−C). Additionally, the UE_{UA} was more strongly correlated with the UE_{GL} than with the plasma AUC of luseogliflozin (Figure 4D, E). Recent studies have demonstrated clearly that a uricosuric effect of several drugs, such as benzbromarone, probenecid, the angiotensin II receptor blocker, losartan, and salicylic acid, are due to the inhibition of URAT1, which is expressed at the apical membrane
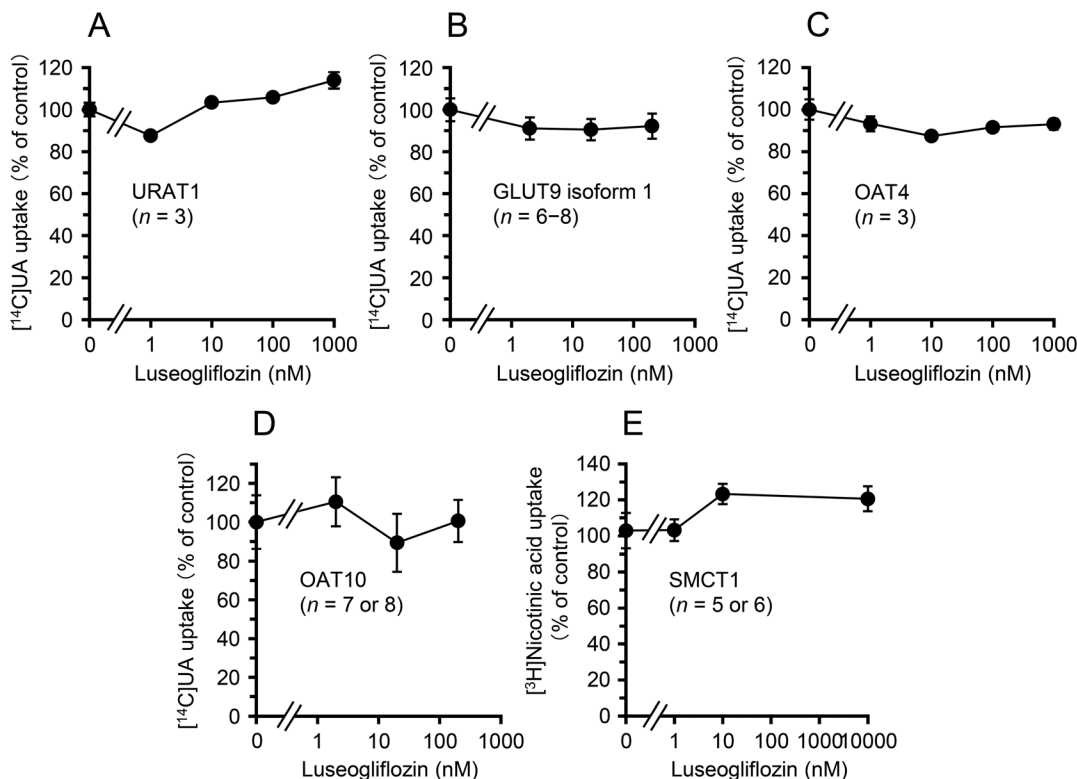
Figure 5. Effect of luseogliflozin on transporters involved in renal uric acid handling in humans. URAT1 (A) and OAT4 (C) were examined in gene-expressing HEK293 and S2 cells, respectively. GLUT9 isoform 1 (B), OAT10 (D) and SMCT1 (E) were examined in gene-expressing *Xenopus* oocytes. Data are mean ± SEM

of tubular epithelial cells and takes up uric acid from the lumen to the cells [24,32]. In addition, several other transporters are considered to be involved in the reabsorption of uric acid, including GLUT9 isoform 1, which plays a predominant role in the basolateral efflux transport of uric acid into the blood side [30,31]; OAT4 and OAT10, which are thought to contribute to uric acid reabsorption at the apical membrane [26,27] and SMCT1, which provides a driving force for URAT1, for the uptake of monocarboxylic acids into the cells [43,44]. However, none of these transporters were affected by luseogliflozin at a clinically attained concentration (Figure 5). Additionally, although a reduction in the serum uric acid has been reported in patients with familial renal glycosuria associated with mutations in SGLT2 [47], inhibition of the reabsorption of uric acid by SGLT2 was excluded. Thus, the uricosuric effect of luseogliflozin could be attributed to the effect of glycosuria, but not to the direct effect of luseogliflozin on uric acid handling in the kidney.

Finally, the study examined whether glycosuria causes an increase in the UE_{UA}. The uricosuric effect of luseogliflozin was correlated with the UE_{GL}-enhancing effect (Figure 4D), and a non-selective SGLT inhibitor, phloridzin, and D-glucose have been suggested to modulate uric acid handling in the proximal tubule [11,35,37]. Accordingly, the study focused on the contribution of GLUT9, which reportedly transports both uric acid and D-glucose [30,31,48]. GLUT9 is a member of the facilitative glucose transporter family and is expressed primarily in the liver and kidney [48]. GLUT9 appears to be involved in the efflux of uric acid [30,31] and has two splice variants [48]. GLUT9 isoform 1 is localized at the basolateral membrane and is essential for uric acid reabsorption together with URAT1 [42,48]. A functional analysis has indicated that uric acid transport mediated by the GLUT9 isoform 1 was *trans*-stimulated by 5 mM of D-glucose [31]. On the other hand, GLUT9 isoform 2 (GLUT9ΔN, *SLC2A9b*) is localized at the apical
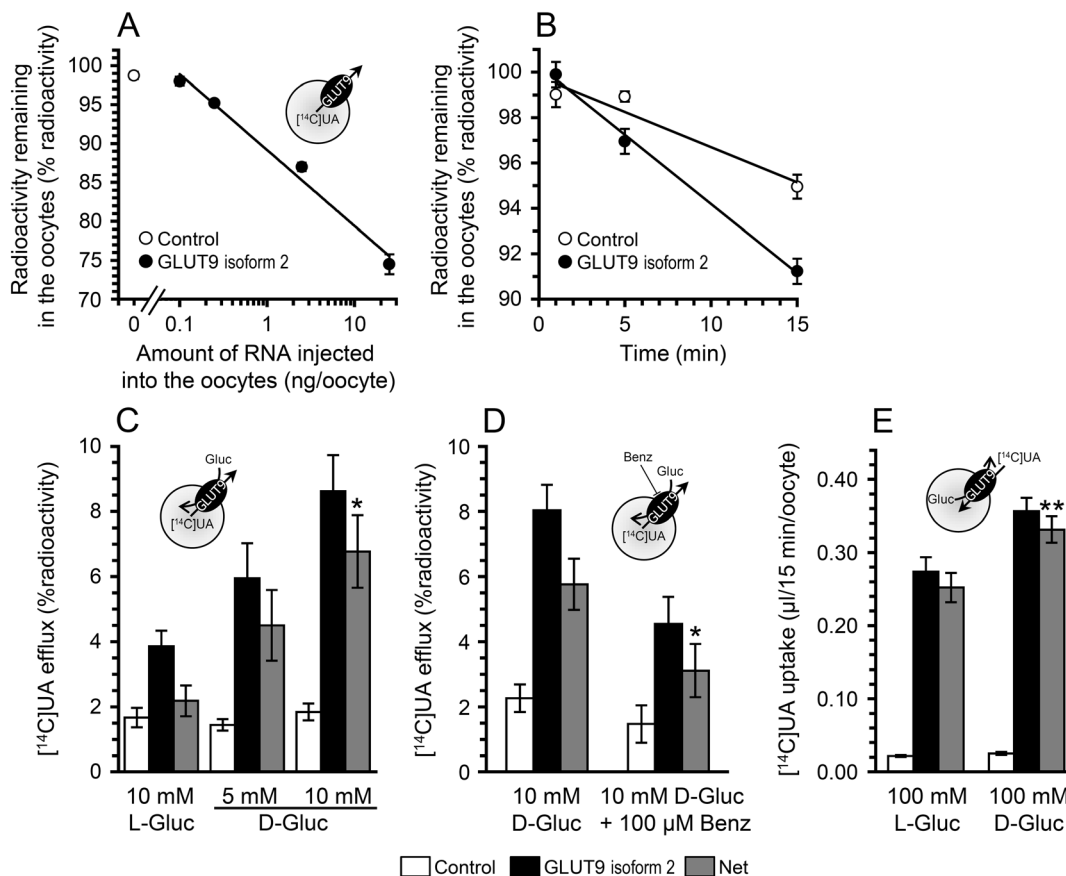
Figure 6. *Trans*-stimulatory effect of D-glucose on uric acid transport by GLUT9 isoform 2. Effect of the injected amount of cRNA of GLUT9 isoform 2 into *Xenopus* oocytes on UA efflux (n = 7–8) determined at 5 min and (B) time-course of UA efflux from the oocytes injected with 0.25 ng of cRNA (closed circle) or water (open circle) (n = 4–8). (C) *Trans*-stimulatory effect of D-glucose (n = 5–10) and (D) *cis*-inhibitory effect of benzbromarone (Benz) (n = 7–10) on GLUT9 isoform 2-mediated [14C]UA efflux. (E) *Trans*-stimulatory effect of D-glucose on GLUT9 isoform 2-mediated [14C]UA uptake (n = 14–17). The efflux and uptake of [14C]UA were evaluated using oocytes injected with 0.25 ng and 25 ng of cRNA, respectively. Data are mean ± SEM. *p < 0.05 vs 10 mM L-glucose (C, Dunnett's test) or vs 10 mM D-glucose (D, Aspin-Welch's *t*-test), **p < 0.01 vs 100 mM L-glucose (E, Aspin-Welch's *t*-test)

membrane [42,48], but its physiological role is unknown. Reportedly, D-glucose does not stimulate uric acid transport mediated by the GLUT9 isoform 2 at a concentration of ~5 mM [30,49]. However, it was hypothesized that the GLUT9 isoform 2 is stimulated by D-glucose at concentrations higher than 5 mM, since it had not been examined at these concentrations. Additionally, the two isoforms differ only in the *N* terminal [48] and their transport functions of uric acid and hexoses are similar [30,31]. In healthy humans, the average plasma glucose level is 5–5.5 mM and increases up to approximately 10 mM after food intake [4]. Most of the plasma glucose is filtered at the glomerulus and the filtrate is concentrated in the proximal tubule.

Because SGLT2 inhibitor blocks SGLT2 localized at the S1 segment in the beginning of the proximal tubule, the glucose level in this part is likely to be higher than 5 mM.

To address this possibility, the study examined the effect of 5 mM and a higher concentration of D-glucose on [14C]UA efflux mediated by GLUT9 isoform 2 using *Xenopus* oocytes expressing the transporter. As a result, a significant stimulation of [14C]UA efflux by D-glucose was observed at a concentration of 10 mM (Figure 6C). The contribution of GLUT9 isoform 2 to this enhanced uric acid efflux was confirmed by the inhibition of a high concentration of benzbromarone [29–31] (Figure 6D). In addition, it was observed that D-glucose stimulated
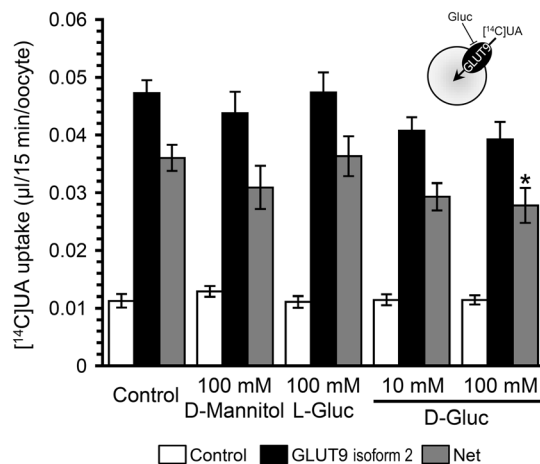
Figure 7. *Cis*-inhibitory effect of D-glucose on GLUT9 isoform 2-mediated [^{14}C]UA uptake in oocytes injected with 25 ng of cRNA ($n = 8$–12). Data are mean ± SEM. *$p < 0.05$ vs control (transport buffer) (Aspin-Welch's *t*-test), Significant difference was not observed as a result of multiple comparison (Dunnett's test) was used
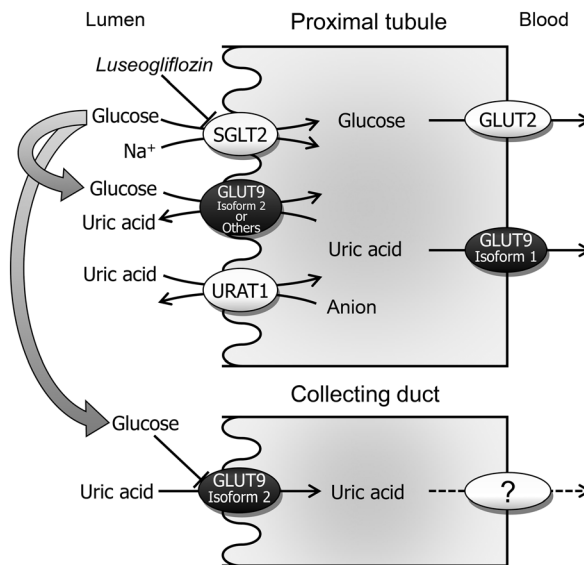

Figure 8. Proposed model for uricosuric effect of SGLT2 inhibitors by glycosuria-induced uric acid secretion via GLUT9 isoform 2 or any other functionally similar transporters at the proximal tubule and inhibition of uric acid uptake via GLUT9 isoform 2 at the collecting duct of renal tubule

uric acid transport by GLUT9 isoform 2 in the opposite direction (Figure 6E). Our data are consistent with a previous study that showed a negative stimulatory effect of D-glucose on both uric acid efflux and uptake [30,49]. In our *in vitro* experiments, the stimulatory effect was not stably reproducible when 25 ng cRNA/oocyte was injected. However, the stimulation effect became reproducible when the efflux activity of GLUT9 was lowered by decreasing the injected amount of cRNA to 0.25 ng/oocyte. The observation suggests that the intracellularly injected uric acid was rapidly effluxed by the GLUT9 isoform 2 when it is sufficiently expressed by the *in vitro* experiment. In addition, since GLUT9 is thought to be an efflux transporter of anionic uric acid using a negative membrane potential [30], uric acid uptake was examined under degraded membrane potential conditions. As a result, the basal uric acid uptake was increased and a D-glucose-induced stimulation of uric acid efflux was observed. Very recently, Kimura *et al*. reported the expression of GLUT9 isoform 2 protein in the collecting ducts and the possibility of its contribution to uric acid reabsorption [50]. Urinary concentrations of glucose higher than 100 mM were observed clinically after the administration of luseogliflozin at doses of > 3 mg

(data not shown). As a result, uric acid uptake by GLUT9 isoform 2-expressing oocytes was inhibited by D-glucose at 100 mM (Figure 7). Based on these results, one can conclude that the increased concentration of glucose in the lumen by SGLT2 inhibition stimulates uric acid excretion mediated by GLUT9 isoform 2 or any other transporter(s) in the proximal tubule and inhibits uric acid reabsorption mediated by GLUT9 isoform 2 in the collecting duct (Figure 8).

The serum uric acid level is closely related to markers of insulin resistance [38]. Insulin has been suggested to increase uric acid reabsorption with an increased Na$^+$ reabsorption in the proximal tubule [34]. On the other hand, the serum uric acid level of patients with type 2 diabetes is known to be lower than that of the normal population [35,37,38], and the CL_{UA} of the patients is higher than that of the normal population [37]. Moreover, the SUA reportedly increases and the UE_{UA} decreases after glycemic control for the treatment of diabetes [51]. In addition, UE_{UA} is induced by glycosuria after D-glucose infusion [11,52]. Until now, the decrease of SUA in patients with type 2 diabetes has been explained by osmotic diuresis caused by glucose [11,51], an alteration in the proximal tubule [51] and/or the effect of glucose

on renal uric acid handling [11,37], especially the competitive inhibition of uric acid reabsorption [35], although the exact mechanisms remain unclear. The present observations of the decrease in the SUA level and the increase in the UE_{UA} in association with an increase in the UE_{GL} are in accordance with previous reports. Additionally, the *in vitro* experimental results suggested that the luminal glucose possibly stimulates the UE_{UA} via the GLUT9 isoform 2 or any other transporter(s). Based on these results, the mechanism of SUA reduction in patients with type 2 diabetes may also be explained by the same mechanism.

In summary, the present study demonstrated that the SUA-lowering effect of SGLT2 inhibitor can be accounted for by the increase in UE_{UA}. The increase in UE_{UA} is not due to a direct effect of the drug on the renal handling of uric acid, but may be attributed to glycosuria caused by SGLT2 inhibitors on the GLUT9 isoform 2 or any other transporter(s) at the proximal tubule and may inhibit uric acid reabsorption mediated by GLUT9 isoform 2 at the collecting duct of the renal tubule. In addition, this mechanism may also explain the relation between glycosuria and the reduction in the SUA observed in patients with type 2 diabetes.

Acknowledgements

Conflicts of Interest

Yukihiro Chino, Yoshishige Samukawa, Soichi Sakai, Yasuhiro Nakai and Jun-ichi Yamaguchi are employees of Taisho Pharmaceutical. No other potential conflicts of interest relevant to this article are reported.

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