Reduction of Renal Transport Maximum for Glucose by Inhibition of Na$^+$-Glucose Cotransporter Suppresses Blood Glucose Elevation in Dogs

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T-1095, an orally active inhibitor of Na$^+$-glucose cotransporter (SGLT), excretes excess plasma glucose into urine, lowers blood glucose levels, and thus has therapeutic potential for treatment of diabetes mellitus. To elucidate the correlation between threshold for renal glucose reabsorption and blood glucose levels, we evaluated the effects of T-1095 on transport maximum for glucose (TmG) in dogs. Intravenous infusion of T-1095A (0.25—2.0 µg/kg/min), an active metabolite of T-1095, dose-dependently increased fractional glucose excretion induced by a hyper-amount of glucose infusion in anesthetized dogs. Calculated TmG was decreased by T-1095A in a dose dependent manner, and plasma concentration of T-1095A correlated well with the reduction of TmG ($R^2=0.704$). Then, oral glucose tolerance tests (OGTT) were carried out in dogs. T-1095 at a dose of 3 mg/kg (*p.o.*) slightly increased urinary glucose excretion without affecting blood glucose levels. Ten mg/kg (*p.o.*) of T-1095 suppressed the elevation of blood glucose levels by excreting a large quantity urinary glucose. The estimated TmG reduction by 3 and 10 mg/kg of T-1095 was about 50% and more than 80%, respectively. In conclusion, this study clarified that more than 80% reduction of TmG by inhibition of SGLT was necessary for suppressing postprandial hyperglycemia in normoglycemic dogs.

Key words   T-1095; Na$^+$-glucose cotransporter (SGLT); transport maximum for glucose (TmG); glucose tolerance test; dog; antidiabetic agent

Several oral antidiabetic drugs and insulin have been available for the treatment of diabetes at present. Although each drug may be highly effective for some patients, it is still difficult to maintain good glycemic control in most diabetic patients.[1] Recently, we have proposed the inhibition of renal Na$^+$-glucose cotransporter (SGLT) as a new therapeutic strategy for diabetes.[2,3]

Previously, we have identified a novel SGLT inhibitor, 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-β-D-glucopyranoside (T-1095A). Its acylated prodrug, 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-(6-*O*-methoxycarbonyl)-β-D-glucopyranoside (T-1095) is an orally active compound.[3] T-1095 treatments reduced blood glucose levels both in type 1[2,4,5] and type 2[2,6—9] diabetic animal models.

The antidiabetic effects of T-1095 are based on the excretion of excess plasma glucose into urine. Single administration of T-1095 increases glucose excretion by inhibiting renal glucose reabsorption, and continuous treatment of T-1095 corrects elevated blood glucose levels.[2,6—9] Plasma glucose is filtered in the glomerulus of kidney and filtered glucose is reabsorbed in proximal tubules. This process of glomerular filtration and tubular reabsorption of glucose depends on the plasma glucose levels.[10] Therefore, when the blood glucose levels is reduced by the SGLT inhibitor, the glomerular glucose filtration is decreased. Consequently, the reduction of filtrated glucose leads to decrease in urinary glucose excretion. Thus, urinary glucose excretion is the main mechanisms of T-1095 but the amount of urinary glucose does not directly reflect the status of SGLT inhibition.

In order to clarify the relation of SGLT inhibition and glucose reabsorption capacity, we investigated the *in vivo* effect of the SGLT inhibitor in glucose-loaded hyperglycemic conditions and during an oral glucose tolerance test (OGTT) in dogs using transport maximum for glucose (TmG) as a quantitative index.

MATERIALS AND METHODS

Chemicals T-1095A and its acylated prodrug T-1095 were synthesized at the Medicinal Chemistry Research Laboratories in Tanabe Seiyaku Co., Ltd. T-1095A was dissolved in polyethylene glycol 400 and was diluted with physiological saline for an intravenous infusion. The final concentration of polyethylene glycol 400 was 1%. T-1095 was suspended in 0.1% (w/v) hydrogenated castor oil polyethylene glycol ester (Nikkol® HCO-60, Nikko Chemicals, Tokyo, Japan) for an oral administration. All other chemicals used were of guaranteed reagent grade.

Animals Mongrel dogs of either sex were purchased from KBL Yoshiki Farm (Gifu, Japan). They were housed in individual cages, and allowed free access to tap water. Standard laboratory chow (DS-5, Oriental Yeast, Tokyo, Japan) of 30 g/kg was given every morning. The animal rooms were controlled for temperature (23±2 °C), humidity (55±5%) and light (12 h light–dark cycle). Experimental protocols concerning the use of laboratory animals were reviewed and approved by the Tanabe Seiyaku Co., Ltd. Ethics Committee.

Effect of T-1095A on Renal Glucose Reabsorption (Experiment 1) Twenty five mongrel dogs of either sex (body weight, 12.0—20.3 kg) were anesthetized with an intravenous administration of sodium pentobarbital at a dose of 30 mg/kg. A cuffed endotracheal tube was inserted, and the animal was artificially ventilated with room air at a volume of 15 ml/kg and a rate of 18 strokes/min. The right brachial artery, right femoral artery, left cephalic vein, and each branch of the left and right femoral veins were cannulated for a collection of blood samples, measurement of arterial pressure, drug administration, and infusion of anesthetics, creatinine and glucose, respectively. Continuous intravenous infusion of sodium pentobarbital at a rate of 5—6 mg/kg/h maintained anesthesia throughout the experiment. Warmed saline at 37 °C containing 0.5% (w/v) creatinine was given intra-

venously at a volume of 5 ml/kg, and then warmed saline containing 0.3% (w/v) creatinine was infused at a rate of 0.05 ml/kg/min. The left kidney was exposed by an incision in the retroperitoneal flank. An electromagnetic flow probe was placed on the left renal artery to monitor the renal blood flow. A catheter for urine collection was inserted into the left ureter. After stabilization of arterial blood pressure, renal blood flow and urine flow, urine samples were collected every 10 min. Blood sampling was conducted at a midpoint of each urine collection period. During the course of the sampling, glucose was loaded at 20 mg/kg/min (25 μl/kg/min as 80% solution) for 60 min for twice to determine the maximum rate of glucose transport. When the urinary glucose level returned to the baseline after the first glucose load (about 60—150 min), T-1095A infusion was started. The infusion rate of T-1095A was 0, 0.25, 0.5, 1.0 or 2.0 μg/kg/min in each animal. Thirty minutes later, the second glucose load was carried out. Blood samples were transferred to chilled tubes containing heparin, and plasma was obtained to measure the concentration of creatinine, glucose and T-1095A.

Effect of T-1095 in OGTT (Experiment 2) Six male mongrel dogs (body weights, 14.7—18.7 kg) were used. Two series of crossover studies were performed. One half of animals received the vehicle and another half did T-1095 at 3 mg/kg. One week after, the treatment was exchanged. Three weeks later, the study with 10 mg/kg of T-1095 was carried out by the same procedures. After 24 h fast, animals received orally either vehicle or T-1095 at 15 min before the glucose challenge. An oral glucose load of 2 g/kg was conducted with 40% glucose solution. Blood samples were collected by heparinized syringes from the cephalic vein just before the drug administration, just before the glucose challenge, 15, 30, 60 and 90 min after the glucose challenge to determine blood glucose levels. The blood samples were immediately centrifuged, and plasma was obtained for measurement of T-1095A. Animals were placed in metabolic cages, and 24-h urine was collected to measure urinary glucose excretion. Reduction of TmG was estimated from the

plasma T-1095A concentration and concentration–inhibition relationship that obtained in the Experiment 1.

Measurements Blood glucose levels were determined using commercially available kit based on the glucose oxidase method (New Blood Sugar Test®; Boeringer Mannheim, Mannheim, Germany). Urinary glucose concentration was measured by a Glucose Analyzer (APEC, Inc., Danvers, MA, U.S.A.). Plasma and urinary creatinine contents were measured by an enzymatic method using an automatic analyzer (Hitachi 705, Hitachi, Tokyo, Japan). Plasma T-1095A concentration was determined by an HPLC method. Fractional glucose excretion (FE_{glu}) was calculated from the following equation: $FE_{glu}=[$(urinary flow×urinary glucose concentration)/(creatinine clearance (C_{cr})×plasma glucose concentration)$]×100$. Glucose reabsorption was calculated from the following equation: glucose reabsorption$=[(C_{cr}×$plasma glucose concentration$)-($urinary flow×urinary glucose concentration$)]/$body weight. TmG was defined as glucose reabsorption reached a plateau at 50—60 min after starting the glucose infusion.

Data Analysis Data are expressed as means±S.E.M. Paired t-test was used for comparisons between data for the first and second glucose infusion. Fisher's least-significant difference test was applied to data obtained for the OGTT. Probabilities less than 5% ($p<0.05$) were considered to be statistically significant.

RESULTS

Effect of T-1095A on Renal Glucose Reabsorption Figure 1 indicates changes in FE_{glu} during intravenous glucose loading with or without T-1095A. T-1095A dose-dependently increased FE_{glu}. Two μg/kg/min of T-1095A increased FE_{glu} even without a glucose infusion (at 0 min). FE_{glu} was negligible when plasma glucose concentrations were below 150 mg/dl. FE_{glu} gradually increased over this concentration, which represented the renal threshold for glucosuria (Figs. 1, 2). Only the highest dose of T-1095A sig-
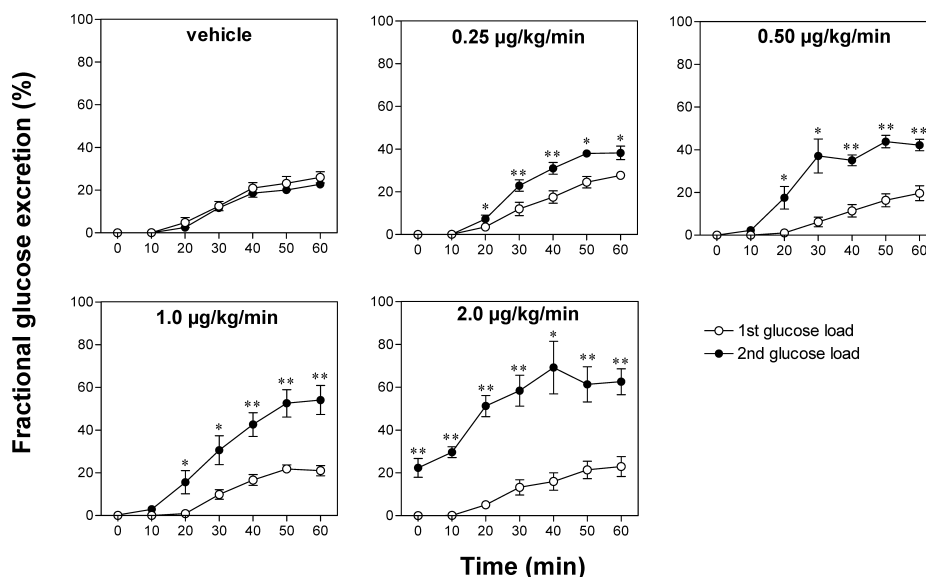

Fig. 1. Effect of T-1095A on Renal Fractional Glucose Excretion during Intravenous Glucose Loading in Anesthetized Dogs

Intravenous infusion of T-1095A was started 30 min before the second glucose load. Each point and vertical bar shows the mean±S.E.M. of 5 experiments. *$p<0.05$ and **$p<0.01$ as compared with the corresponding values of the first glucose load by paired t-test.
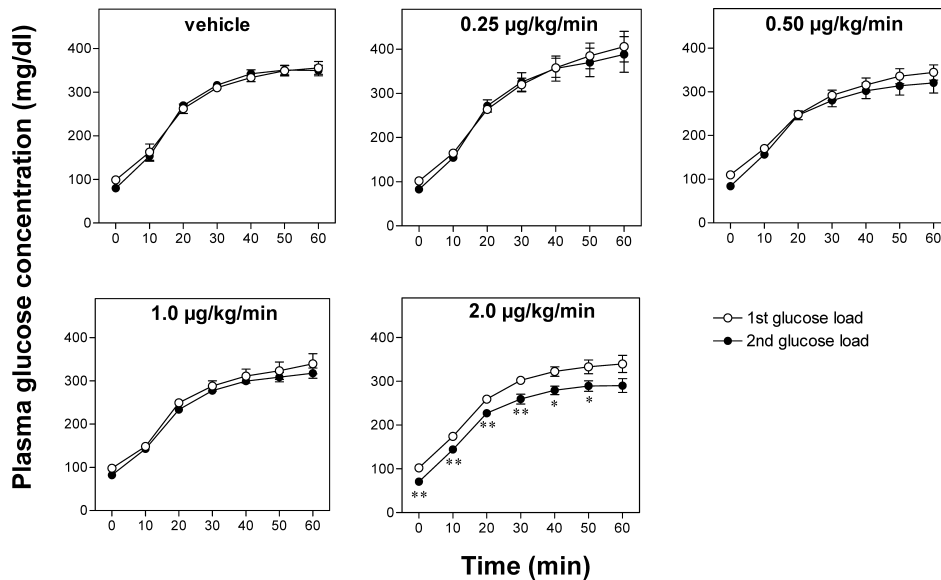
Fig. 2.   Effect of T-1095A on Plasma Glucose Concentration during Intravenous Glucose Loading in Anesthetized Dogs

Intravenous infusion of T-1095A was started 30 min before the second glucose load. Each point and vertical bar shows the mean±S.E.M. of 5 experiments. $*p<0.05$ and $**p<0.01$ as compared with the corresponding values of the first glucose load by paired t-test.
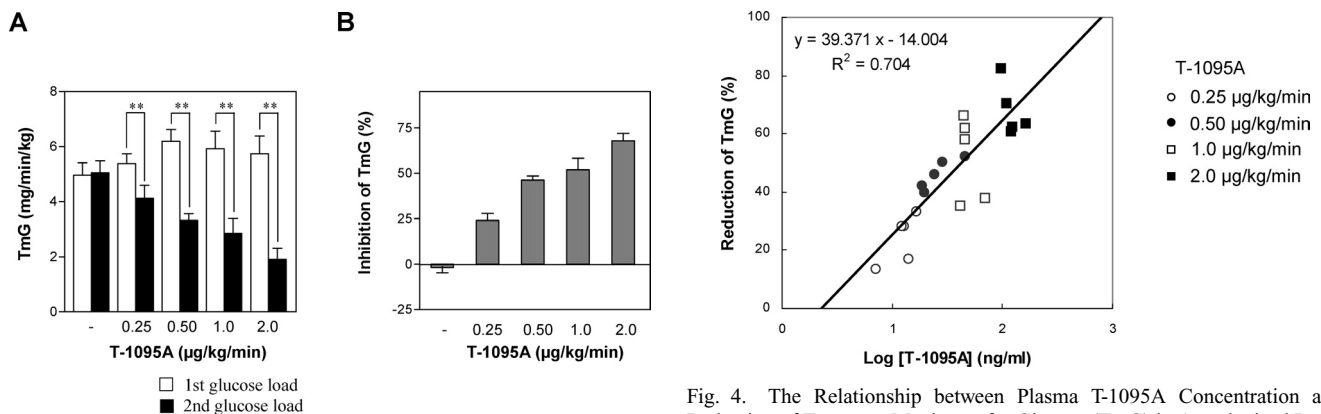

Fig. 3.   Effect of T-1095A on Renal Glucose Transport in Anesthetized Dogs

Values of transport maximum for glucose (TmG, panel A) were determined at 50—60 min of glucose loading. Intravenous infusion of T-1095A was started 30 min before the second glucose load. Percent inhibition of TmG was represented in the panel B. Each column and vertical bar shows the mean±S.E.M. of 5 experiments. $**p<0.01$ as compared with the values of the first glucose load by paired t-test.

Fig. 4.   The Relationship between Plasma T-1095A Concentration and Reduction of Transport Maximum for Glucose (TmG) in Anesthetized Dogs

nificantly lowered basal glucose concentration and suppressed the increase in plasma glucose concentration during glucose infusion. As shown in Fig. 3, T-1095A decreased TmG in a dose dependent manner. At a dose of 2.0 μg/kg/min T-1095A reduced TmG by approximately 70%. The plasma T-1095A concentration was positively correlated with the reduction of TmG ($R^2=0.704$, Fig. 4). The baseline values of C_{cr} in the left kidney were 1.78±0.07 ml/min/kg ($n=25$). Fractional sodium excretion (FE_{Na}) was slightly decreased only at a dose of 1.0 μg/kg/min at 40 and 50 min after glucose loading (Fig. 5).

Effect of T-1095 in OGTT   Figure 6 shows the results of OGTT in dogs. An oral administration of T-1095 (3 mg/kg) increased urinary glucose excretion (vehicle: 3.1±1.5 vs. 3 mg/kg of T-1095: 87.1±18.6 mg/day/kg, $p<0.01$) without affecting the blood glucose levels (Figs. 6A, C). As shown in Figs. 7A and C, the plasma concentration of T-1095A was

33.3—69.1 ng/ml, and the reduction of estimated TmG was approximately 50% during OGTT (at 0—90 min). T-1095 at a dose of 10 mg/kg markedly increased urinary glucose excretion (vehicle; 1.5±0.5 vs. 10 mg/kg of T-1095; 775.4±72.7 mg/day/kg, $p<0.01$), and suppressed blood glucose elevation after glucose challenge (Figs. 6B, D). The plasma concentration of T-1095A was 60.2—483.1 ng/ml, and the estimated TmG was decreased by 53.3—88.8% at 0—90 min after glucose loading (Figs. 7B, D).

DISCUSSION

Plasma glucose is filtered in glomeruli and then reabsorbed in epithelial cells of renal proximal tubules via SGLT.[10,11] As we have reported previously, T-1095, an orally active inhibitor of SGLT, excretes excess plasma glucose into urine, lowers blood glucose levels,[2—9,12] and thus has therapeutic potential for treatment of diabetes mellitus. However, to date, it is not well understood how much reduction of threshold for renal glucose reabsorption is needed for decreasing blood glucose levels. Therefore, in the present study, we evaluated the effects of T-1095 on both reduction of trans-
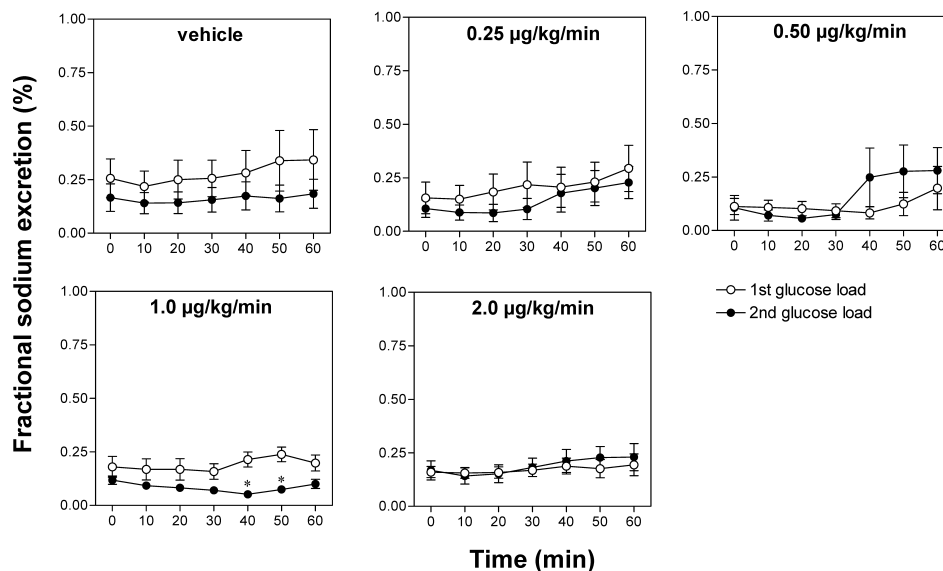
Fig. 5. Effect of T-1095A on Fractional Sodium Excretion during Intravenous Glucose Loading in Anesthetized Dogs

Intravenous infusion of T-1095A was started 30 min before the second glucose load. Each point and vertical bar shows the mean±S.E.M. of 5 experiments. *$p<0.05$ as compared with the corresponding values of the first glucose load by paired t-test.
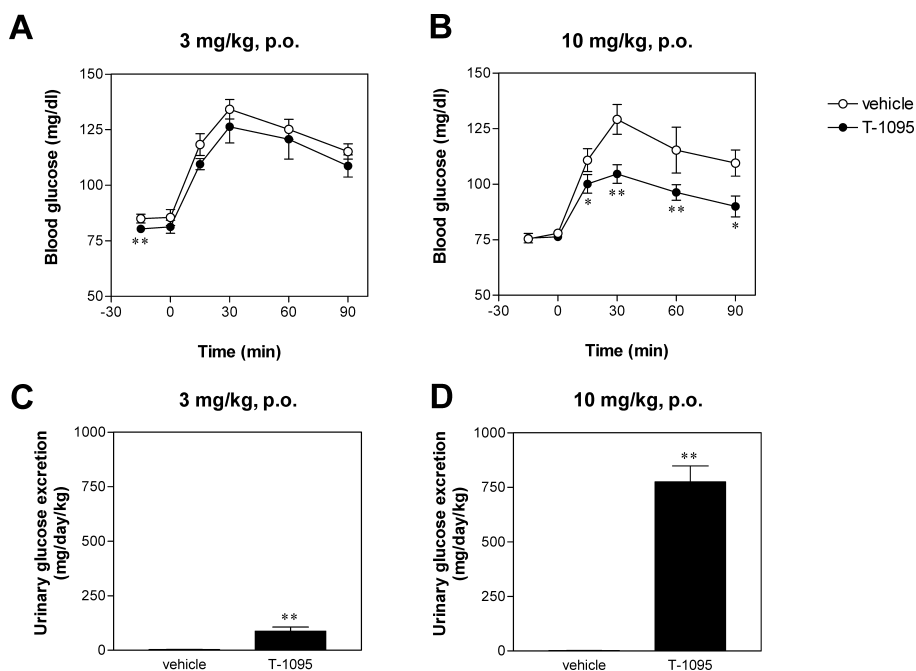

Fig. 6. Effect of T-1095 in Oral Glucose Tolerance Tests in Dogs

Blood glucose (A, B) and urinary glucose excretion (C, D) are represented. T-1095 was orally administered at 15 min before the glucose challenge. Each point and vertical bar shows the mean±S.E.M. of 6 experiments. *$p<0.05$ and **$p<0.01$ as compared with the corresponding values of vehicle group by Fisher's least-significant difference test.

port maximum for glucose (TmG) and lowering of blood glucose levels in dogs.

In the present study, the calculated glucose filtration rate at a baseline was 1.82 ± 0.08 mg/kg/min ($n=25$) in the left kidney. It was only 32% of TmG (5.64 ± 0.23 mg/kg/min, $n=25$) in normal dogs. In other words, the tubular system possesses approximately 3-fold capacity for glucose reabsorption at a normoglycemic range. These results are consistent well with those of Deetjen et $al.$[11]

In this study, T-1095A increased FE_{glu}, and decreased TmG under hyperglycemic states induced by glucose infusion (Figs. 1—4). The basal plasma glucose levels and

plasma glucose increments were reduced by only the highest dose of T-1095A with more than 70% inhibition of TmG under these condition (Figs. 2, 3). During OGTT, blood glucose rose to about 130 mg/dl in dogs. The blood glucose elevation was suppressed with more than 80% reduction of TmG by T-1095 at a high dose (10 mg/kg). However, there was no suppression of blood glucose elevation when slight glucosuria was induced by 50% reduction of TmG (3 mg/kg). These results clearly demonstrate that more than 70—80% reduction of TmG by inhibition of SGLT is necessary for suppressing postprandial hyperglycemia in normal dogs.

Na^+ is reabsorbed together with glucose by SGLT in the

A 3 mg/kg, p.o.

B 10 mg/kg, p.o.
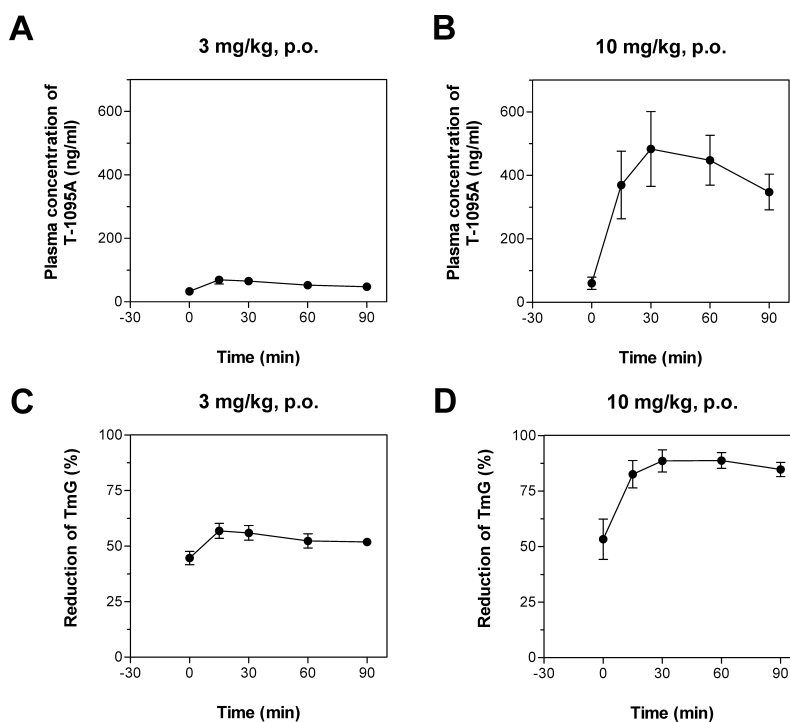
C 3 mg/kg, p.o.

D 10 mg/kg, p.o.

Fig. 7. Effect of T-1095 in Oral Glucose Tolerance Tests in Dogs

Plasma T-1095A concentration (A, B) and percent reduction of transport maximum for glucose (C, D) are represented. T-1095 was orally administered at 15 min before the glucose challenge. Each point and vertical bar shows the mean±S.E.M. of 6 experiments.

proximal tubule. In this study, Na^+ excretion was only slightly affected at a dose of $1.0\,\mu g/kg/min$ of T-1095A treatment (Fig. 5). There are many types of Na^+-coupled transporters other than SGLT in renal tubules, and Na^+ would be reabsorbed by these transporters.[10] We have also reported that T-1095 does not increase Na^+ content of urine, nor affect plasma osmolarity and the contents of electrolytes in the diabetic KK-A^y mice.[3] Thus selective SGLT inhibitor does not seem to influence the electrolyte balance in plasma and urine.

In conclusion, present study clearly demonstrated that the relationship between reduction of TmG and the plasma concentration of an SGLT inhibitor, T-1095A in normal dogs *in vivo*. In normoglycemic conditions, more than 80% of reduction of TmG by inhibition of SGLT was needed for decreasing blood glucose levels.

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