T-1095, a Renal Na^+-Glucose Transporter Inhibitor, Improves Hyperglycemia in Streptozotocin-Induced Diabetic Rats

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The effect of T-1095, an inhibitor of renal glucose reabsorption, on hyperglycemia and the expression of Na^+-glucose cotransporters (SGLTs) and facilitative glucose transporter 2 (GLUT2) in streptozotocin (STZ)-induced diabetic rats was examined. There was an elevation of blood glucose, hemoglobin A_{1c} (HbA_{1c}), kidney weight, and urinary excretion of both glucose and albumin in STZ rats. Administration of 0.03% and 0.1% (wt/wt diet) T-1095 to STZ rats for 4 weeks improved the hyperglycemia and dose-dependently decreased HbA_{1c}. Moreover, treatment with 0.1% (wt/wt diet) T-1095 in STZ rats for 8 weeks not only reduced blood glucose and HbA_{1c} levels but also prevented the elevation of urinary albumin levels and kidney weight and the development of epithelial vacuolation. The expression of renal SGLT2, a major glucose transporter in the kidney, was not different in normal, STZ, and T-1095-treated STZ rats. In contrast, the elevated renal GLUT2 level in STZ rats was suppressed by T-1095. These data suggest that T-1095 improves hyperglycemia by suppressing the renal reabsorption of glucose, which results in a suppression of the development of functional and histological changes and abnormal expression of GLUT2 in the kidney.

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THE MAJOR RENAL glucose transporter, Na^+-glucose cotransporter 2 (SGLT2), is present primarily in the proximal tubule S1 segment, where it reabsorbs almost all of the glucose.[1] SGLT1 is present primarily in the proximal tubule S3 segment and reabsorbs the glucose that has not been reabsorbed in the S1 segment,[2] and is also present in the epithelial membrane of the small intestine and absorbs glucose.[3,4] GLUT2, one of the facilitative glucose transporters, occurs in the basolateral membrane of renal tubules and intestinal basolateral membrane, and the glucose that is absorbed by SGLTs leaves the enterocyte by moving across the basolateral membrane.[3-6]

Phlorizin inhibits SGLTs and suppresses renal glucose reabsorption, resulting in an increased urinary excretion of glucose and a reduction of plasma glucose levels in diabetic model animals.[7] However, phlorizin with oral administration is poorly absorbed in the small intestine and is almost entirely degraded to phloretin, which inhibits facilitative glucose transporters,[8-10] making it an unlikely appropriate oral therapeutic agent for the inhibition of renal glucose reabsorption. We have produced T-1095, a prodrug (Fig 1) that is converted to its active metabolite in vivo and is a selective and potent inhibitor of renal SGLTs.[11,12]

In this study, we examined the effects of T-1095 on glycemic control in streptozotocin (STZ)-induced diabetic rats without insulin treatment. We also examined whether T-1095 treatment suppresses the development of renal histological changes, because glomerular hypertrophy and vacuolation of renal tubules occur in diabetes.[13-15] In addition, we examined the effect of T-1095 on the expression of SGLTs and GLUT2 in the kidney and small intestine.

MATERIALS AND METHODS

Animals and Study Design

Male Wistar rats (aged 7 weeks; Japan SLC, Shizuoka, Japan) at 1 week after intravenous injection of STZ (50 mg/kg body weight in 100 μL 50-mmol/L sodium citrate, pH 4.5) were used. STZ-treated rats were fed a normal diet (CE-2; Clea Japan, Osaka, Japan) for 4 weeks (n = 10) or 8 weeks (n = 4), or a diet of 0.03% or 0.1% wt/wt T-1095 (T-1095 synthesized in the Discovery Research Laboratory, Tanabe Seiyaku, Saitama, Japan) for 4 weeks (0.03%, n = 3; 0.1%, n = 5) or 8 weeks (0.1%, n = 4). Control rats (administered only 100 μL 50-mmol/L sodium citrate, pH 4.5) were fed a normal diet for 4 weeks (n = 10) or 8 weeks (n = 4), ad libitum. The rats were kept at controlled temperature (23° ± 1°C), humidity (55% ± 5%), and lighting (from 7 AM to 7 PM). Plasma glucose was determined with an enzymatic assay kit (Blood Glucose Test; Boehringer, Mannheim, Germany). Hemoglobin A_{1c} (HbA_{1c}) was determined by an aminophenyl-boronate-agarose affinity chromatographic method (Glyc-Affin GHb; Seikagaku Kogyo, Tokyo, Japan). The urine of each rat was collected for 24 hours with a metabolic cage, and the urinary glucose level and albumin content were measured with a Glucose Analyzer (APEC, Denver, MA) and an enzyme-linked immunosorbent assay kit (Panatest rat albumin; Panafarmlaboratories, Kumamoto, Japan), respectively.

Normal (n = 5), STZ (n = 4), and 0.1% (wt/wt) T-1095-treated (n = 4) rats fed for 8 weeks were anesthetized with diethylether and killed. The kidneys of each rat were removed and weighed. The left kidney was rapidly frozen in liquid nitrogen and stored at −80°C for immunoblot or Northern blot analysis. The right kidney was fixed in Carnoy's solution and embedded in paraffin, and sections (3 μm thick) were stained with hematoxylin and eosin (HE) or periodic acid Schiff (PAS). The jejunum was cut transversely into 2 segments of roughly equal length. The first segment was flushed with ice-cold phosphate-buffered saline, frozen in liquid nitrogen, and stored at −80°C for

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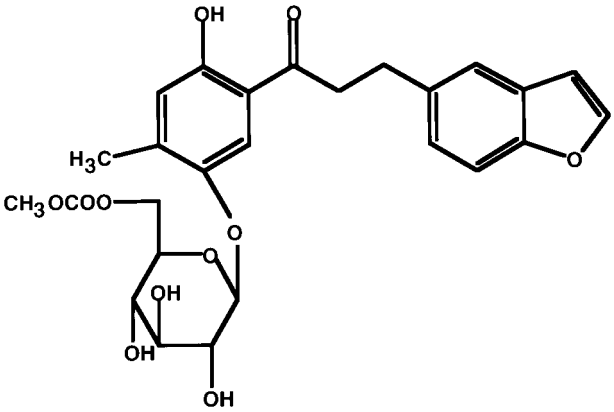

Fig 1. Structure of T-1095.

immunoblot or Northern blot analysis. The other segment was fixed in 10% formaldehyde solution and embedded in paraffin, and sections (3 μm thick) were stained with HE.

Immunoblot Analysis

A part of the kidney or intestine was homogenized in mannitol/HEPES-Tris buffer (100 mmol/L mannitol and 10 mmol/L HEPES-Tris, pH 7.0). Each homogenate was centrifuged at 3,000 × *g* for 10 minutes, and a crude membrane was collected by centrifugation at 100,000 × *g* for 60 minutes. The protein content of the crude membrane was determined with the method described by Lowry et al.[16] A crude membrane (10 μg protein) fraction was subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (10% gel) according to the Laemmli method,[17] and proteins in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was treated with a rabbit antiserum against rat SGLT1 or GLUT2 (Santa Cruz Biotechnology, Santa Cruz, CA) and then with alkaline phosphatase–conjugated goat anti-rabbit antibodies. Immunoreactive proteins were detected for staining with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt, and the relative amount was estimated by scanning densitometry.

Northern Blot Analysis

Total RNA was extracted from the kidney and intestine by the guanidinium thiocyanate cesium chloride method.[18,19] Twenty micrograms of total RNA was denatured in glyoxal and dimethyl sulfoxide and separated by electrophoresis through a 1% agarose gel, and then transferred to a nylon membrane in 20× SSC (3 mmol/L NaCl and 300 mmol/L trisodium citrate). ^{32}P-labeled SGLT1 (625 bp, 1810 to 2434[2]), SGLT2 (570 bp, 1612 to 2181[20]), and GLUT2 (1,485 bp, 1 to 1485[21]) cDNA were synthesized using a commercial kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) and used as probes. The membrane was hybridized overnight at 42°C with 10^6 cpm/mL of the probe in hybridization buffer (final concentration, 50% formamide, 5× SSC, 10% dextran sulfate sodium salt, 0.25% SDS, 20 mmol/L sodium phosphate (pH 7.0), 2× Denhardt reagent,[22] and 100 μg/mL salmon sperm DNA), and β-actin cDNA (287 bp, 862 to 1148[23]) was used for control hybridization. The membranes were washed in 0.1× SSC and 0.1% SDS at room temperature for 10 minutes and then at 50°C for 60 minutes. Hybridized probes on the membranes were detected on an x-ray film and estimated by scanning densitometry.

Statistical Analysis

Statistical evaluations were performed by Student's *t* test. All data are shown as the mean ± SEM, and statistical significance is defined as a *P* value less than .05.

RESULTS

Plasma Glucose, Body Weight, and HbA$_{1c}$

Before administration of T-1095, plasma glucose levels in 0.03% and 0.1% T-1095-treated rats were not different from those in STZ rats (0.03% T-1095 *v* 0.1% T-1095 *v* STZ rats, 22.1 ± 0.5 *v* 22.3 ± 0.4 *v* 22.3 ± 0.2 mmol/L), but plasma glucose in 0.1% T-1095 rats at 4 weeks was significantly lower versus nonSTZ treated rats, and values in 0.03% T-1095 rats were also lower versus nontreated STZ rats, although it was not statistically significant (0.03% T-1095 *v* 0.1% T-1095 *v* STZ rats, 17.0 ± 0.8 *v* 16.8 ± 0.9 *v* 20.4 ± 0.8 mmol/L). Body weight in 0.1% T-1095 rats treated for 4 weeks was significantly higher versus STZ rats (0.03% T-1095 *v* 0.1% T-1095 *v* STZ rats, 160 ± 5 *v* 168 ± 4 *v* 156 ± 5 g). HbA$_{1c}$ levels in T-1095 rats treated for 4 weeks were dose-dependently lower versus STZ rats (0.03% T-1095 *v* 0.1% T-1095 *v* STZ rats, 10.3% ± 0.2% *v* 8.9% ± 0.1% *v* 12.2% ± 0.2%) (Table 1).

After 8 weeks of 0.1% T-1095 administration, plasma glucose and HbA$_{1c}$ levels were significantly lower in 0.1% T-1095 rats versus STZ rats (plasma glucose, T-1095 *v* STZ rats, 15.8 ± 1.3 *v* 26.4 ± 1.2 mmol/L; HbA$_{1c}$, 9.2% ± 0.8% *v* 17.0% ± 0.8%). Body weight in 0.1% T-1095 rats was significantly higher versus STZ rats (T-1095 *v* STZ rats, 189 ± 11 *v* 156 ± 5 g) (Table 2).

Urine Volume, Urinary Albumin and Glucose Excretion, Kidney Weight, and Histological Changes of the Kidney With 8-Week Administration of T-1095

After 8 weeks of T-1095 administration, the urine volume was elevated in STZ rats (96.5 ± 1.9 mL/24 h) and T-1095 rats (97.0 ± 8.7 mL/24 h) compared with normal rats (6.9 ± 0.7 mL/24 h), but there was no significant difference in urine volume between STZ and T-1095 rats. Urinary albumin excre-

Table 1. Body Weight, Plasma Glucose, and HbA$_{1c}$ in Normal, STZ, and 0.03% and 0.1% T-1095-Treated STZ Rats

Parameter	Week	Normal	STZ	0.03% T-1095	0.1% T-1095
Body weight (g)	0	204 ± 1	172 ± 1*	172 ± 5*	174 ± 1*
	4	269 ± 3	156 ± 5*	160 ± 5*	168 ± 4*†
Plasma glucose (mmol/L)	0	5.0 ± 0.1	22.3 ± 0.2*	22.1 ± 0.5*	22.3 ± 0.4*
	4	4.7 ± 0.1	20.4 ± 0.8*	17.0 ± 0.8*	16.8 ± 0.9*†
HbA$_{1c}$ (%)	4	5.2 ± 0.1	12.2 ± 0.2*	10.3 ± 0.2*‡	8.9 ± 0.1*‡

NOTE. Week 0 is 1 week after administration of STZ and before the beginning of administration of T-1095. Data are expressed as the mean ± SEM. Normal rats, n = 10; STZ rats, n = 10; 0.03% T-1095 rats, n = 3; and 0.1% T-1095 rats, n = 5.

*$P < .001$ *v* normal rats.

†$P < .05$, ‡$P < .001$ *v* STZ rats.

Table 2. Body Weight, Plasma Glucose, HbA_{1c}, Kidney Weight, KW/BW, Urine Volume, and Urinary Glucose and Albumin Excretion in Normal, STZ, and T-1095 (0.1%) Rats After 8-Week Administration

Parameter	Normal	STZ	T-1095
Body weight (g)	321 ± 7	156 ± 5†	189 ± 11†‡
Plasma glucose (mmol/L)	5.0 ± 0.1	26.4 ± 1.2†	15.8 ± 1.3†§
HbA_{1c} (%)	5.1 ± 0.1	17.0 ± 0.8†	9.2 ± 0.8†‖
Kidney weight (g)	2.04 ± 0.02	2.45 ± 0.18*	2.18 ± 0.06
KW/BW (%)	0.63 ± 0.01	1.57 ± 0.09†	1.27 ± 0.02†‡
Urine volume (mL/24 h)	6.9 ± 0.7	96.5 ± 1.9†	97.0 ± 8.7†
Urinary glucose excretion (mg/24 h)	4 ± 1	7,546 ± 270†	8,687 ± 336†‡
Urinary albumin excretion (μg/24 h)	450 ± 130	1,649 ± 200†	781 ± 156†‡

NOTE. Data are expressed as the mean ± SEM. Normal rats, n = 5; STZ and T-1095 rats, n = 4.

*$P < .05$, †$P < .001$ *v* normal rats.

‡$P < .05$, §$P < .01$, ‖$P < .001$ *v* STZ rats.

tion was elevated in STZ rats (1,649 ± 200 μg/24 h) compared with normal rats (450 ± 130 μg/24 h) but decreased in T-1095 rats (781 ± 156 μg/24 h). Urinary glucose excretion was significantly higher in T-1095 rats (8,687 ± 336 mg/24 h) versus STZ rats (7,546 ± 270 mg/24 h) (Table 2).

The kidney weight was significantly lower in T-1095 rats (2.18 ± 0.06 g) than in STZ rats (2.45 ± 0.18 g), and kidney weight as a percentage of body weight (KW/BW) was even lower in T-1095 rats (1.27% ± 0.02%) versus STZ rats (1.57% ± 0.09%) (Table 2). Histological sections of the kidney stained with HE are shown in Fig 2. The sections show that epithelial vacuolation of the tubules was less frequent in T-1095 rats versus STZ rats. However, the glomerular tuft surface area was not different in normal, STZ, and T-1095 rats in the sections stained with PAS (data not shown).

Expression Level of Glucose Transporters in Kidney

The expression level of glucose transporters in the kidney is shown in Fig 3. The expression of SGLT2 mRNA was not different in normal, STZ, and T-1095 rats. However, the expression of SGLT1 mRNA and protein was significantly higher in STZ rats versus normal rats, and T-1095 treatment further increased the expression level of SGLT1 mRNA and protein. GLUT2 mRNA and protein were expressed at a significantly higher level in STZ rats than in normal rats, and the expression was significantly lower in T-1095 versus STZ rats.

Effect of T-1095 on Expression Level of Intestinal Glucose Transporters and Histological Changes

The expression level of glucose transporters in the small intestine is shown in Fig 4. The mRNA and protein expression level of SGLT1 and GLUT2 in STZ rats were significantly higher than in normal rats. The expression of SGLT1 and GLUT2 was lower in T-1095 rats than in STZ rats, although it was not of statistical significance, and similar to that in normal rats. In the sections stained with HE in the small intestine, the villus is longer in normal rats versus STZ and T-1095 rats, and there is no histological difference between STZ and T-1095 rats (data not shown).

DISCUSSION

We have previously reported that the newly synthesized agent T-1095 is converted in vivo to its active metabolite and is a selective and potent inhibitor of renal SGLTs and also elicits excretion of excess plasma glucose into the urine.[11,12] In this study, oral administration of T-1095 to insulin-deficient diabetes model STZ rats for 4 weeks dose-dependently decreased HbA_{1c} levels. Moreover, administration of 0.1% T-1095 for 8 weeks clearly decreased plasma glucose and HbA_{1c} levels and sup-

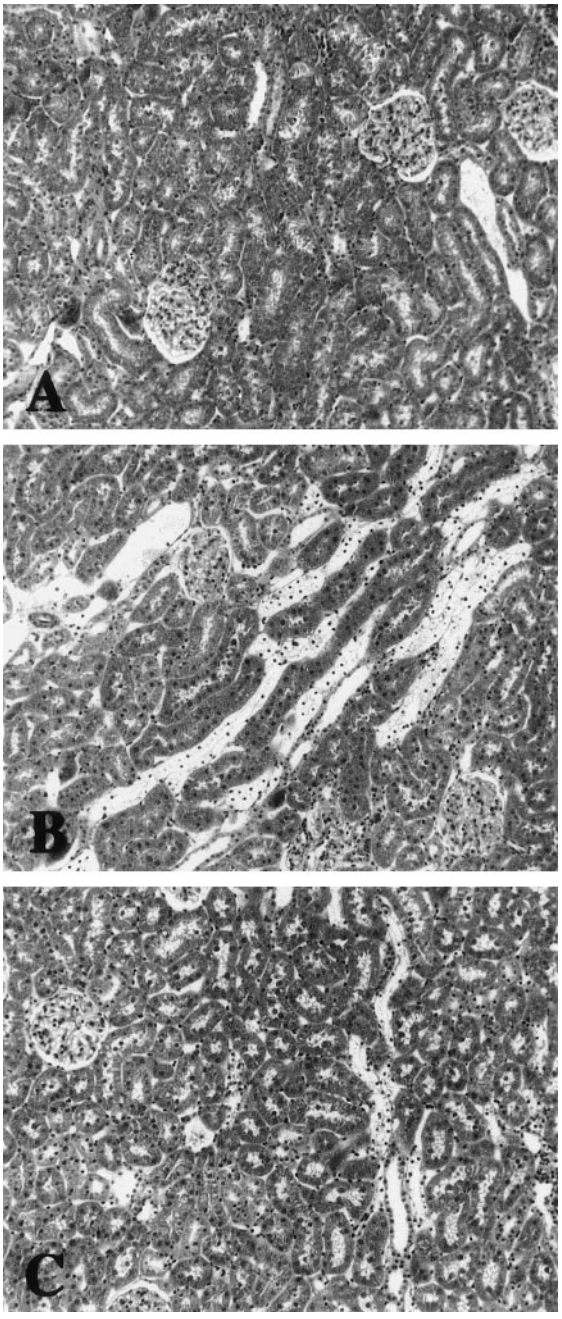

Fig 2. HE-stained histological sections from kidney. Photomicrographs of kidney from normal (A), STZ (B), and T-1095 (C) rats 8 weeks after administration of each diet.

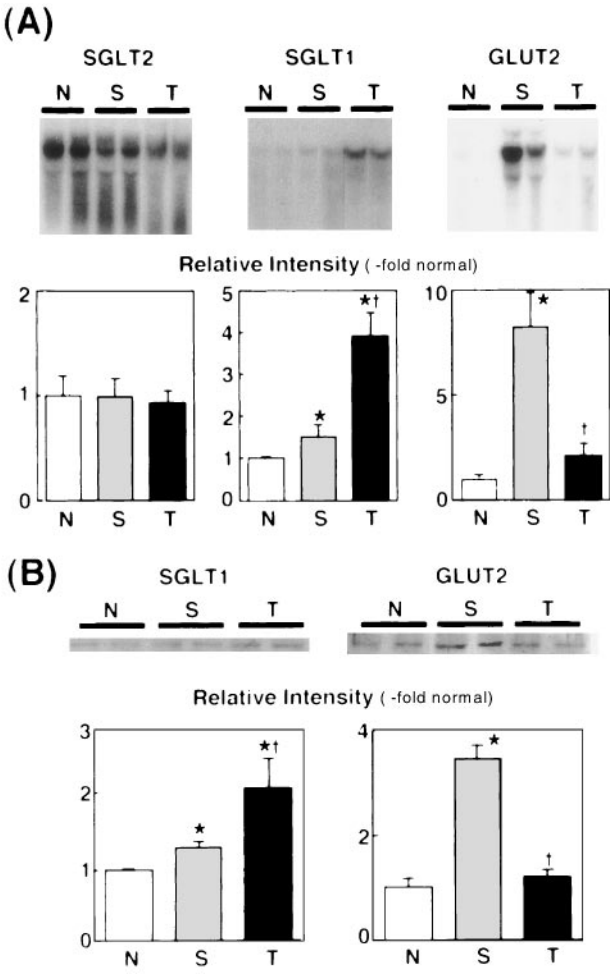

Fig 3. Expression of renal SGLT1, SGLT2, and GLUT2 mRNA (A) and SGLT1 and GLUT2 protein (B) in normal, STZ, and T-1095 rats 8 weeks after treatment, respectively. (A) Northern blot analysis of glucose transporters. A β-actin cDNA served as a control hybridization. Relative intensity of each glucose transporter mRNA expression was calculated against each β-actin mRNA expression, and the expression of normal rats was normalized as 1. (B) Immunoblot analysis of SGLT1 and GLUT2 in each group of rats. Relative intensity of each glucose transporter protein expression was calculated and the expression in normal rats was normalized as 1. Each bar represents the mean ± SEM. N, normal rats; S, STZ rats; T, T-1095 rats. Normal rats, n = 5; STZ rats, n = 4; T-1095 rats, n = 4. *$P < .05$ *v* normal rats, †$P < .05$ *v* STZ rats.

pressed the decrease of body weight. Since oral administration of T-1095 suppressed the body weight loss in nontreated STZ diabetic rats, T-1095 treatment may beneficially affect the metabolic state in diabetes mellitus. Indeed, it has been reported that administration of phlorizin leads to a body weight gain, although it was not statistically significant.[24] However, when phlorizin is administered orally, it is ineffective because it is absorbed poorly and degraded to phloretin in the small intestine, which inhibits facilitative glucose transporters. However, oral administration of T-1095 may be as effective as subcutaneous injection of phlorizin.

Certain histopathological changes in the kidney of STZ-diabetic rats, including an increase of the absolute and relative kidney weight and increased vacuolation of epithelial cells in tubules, were reduced by 0.1% T-1095 treatment for 8 weeks. It has been reported that in STZ-diabetic rats[25,26] and WBN/Kob rats,[27] glycogen granules accumulate in the renal tubules and the amount of glycogen accumulation depends on the plasma glucose concentration.[28] Tubular epithelial cells incorporate glucose from the glomerular filtrate and accumulate glycogen. The vacuoles shown in the histological experiment most nearly represent the glycogen granules in diabetic animals. Since the glucose content in glomerular filtrate is a linear function of the plasma glucose level, the primary effect of T-1095 on glycogen accumulation is likely due to the suppression of the glucose concentration. In addition, there was no sign of histopathological change in the glomerulus while albuminuria was induced

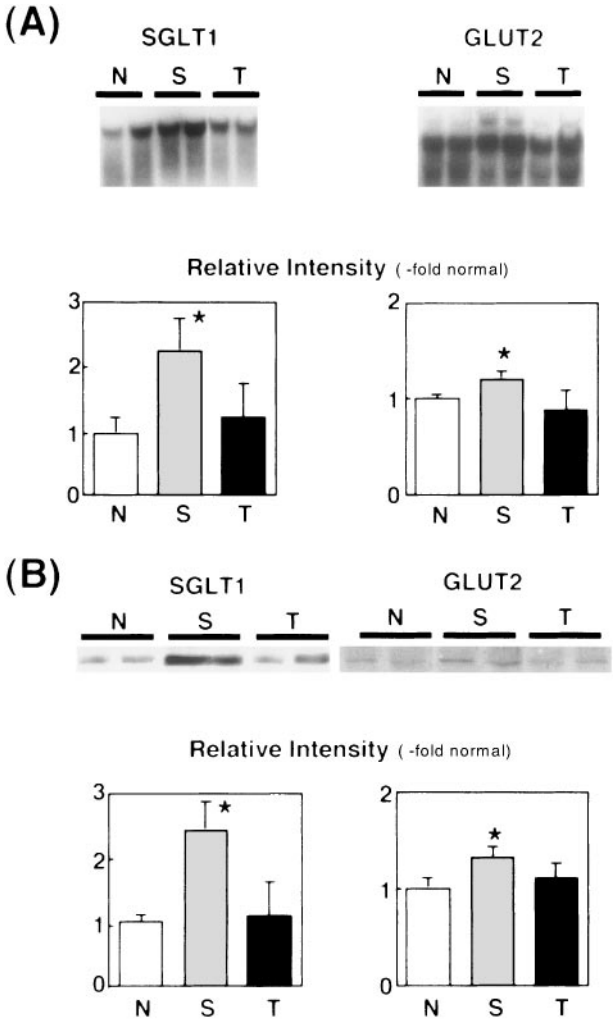

Fig 4. Expression of intestinal SGLT1 and GLUT2 mRNA (A) and protein (B) in normal, STZ, and T-1095 rats 8 weeks after administration. (A) Northern blot analysis of SGLT1 and GLUT2. Relative intensity of each glucose transporter mRNA is calculated as the expression of normal rats to 1. A β-actin cDNA served as a control hybridization. (B) Immunoblot analysis of SGLT1 and GLUT2. Relative intensity of each glucose transporter protein was calculated and the expression of normal rats was normalized as 1. Each bar represents the mean ± SEM. N, normal rats; S, STZ rats; T, T-1095 rats. Normal rats, n = 5; STZ rats, n = 4; T-1095 rats, n = 4. *$P < .05$ *v* normal rats.

during the 8-week diabetic period in STZ-diabetic rats. Because T-1095 treatment reduces urinary albumin excretion and it is likely that the correction of the hyperglycemia limits diabetes-related changes in glomerular function, treatment with T-1095 should improve hyperglycemia, resulting in a suppression of the development of vacuolation of epithelial cells in renal tubules and renal functional changes in STZ rats.

SGLT2 is the major renal glucose transporter and is present in the S1 segment of the proximal tubule, where it reabsorbs almost all of the glucose.[1] SGLT1 is in the S3 segment of the proximal tubule and reabsorbs glucose that has not been reabsorbed in the S1 segment.[2] Regulation of SGLT2 gene transcription has not been characterized, but since the present study has demonstrated that the expression levels of SGLT2 mRNA are similar among normal, STZ, and T-1095-treated STZ rats, it seems unlikely that gene transcription of SGLT2 is related to the plasma or urinary glucose level, and is regulated by T-1095 itself. Expression levels of SGLT1 mRNA and protein are significantly higher in T-1095-treated rats versus normal and STZ rats. The higher total glucose level of the glomerular filtrate observed in STZ-diabetic rats might induce expression of SGLT1 to reabsorb more glucose. Since a much higher level of SGLT1 expression was observed in T-1095-treated STZ rats in which the total urinary excretion of glucose was greater versus STZ rats, the induction of a higher level of SGLT1 in T-1095-treated STZ rats could result from the higher glucose level in the S3 segment. SGLT1 is also found in the epithelial membrane of the small intestine, and it has been reported that glucose transporter levels are altered in STZ-induced diabetic rats.[5,29] In the present study, the expression levels of intestinal SGLT1 mRNA and protein in STZ rats were significantly higher, and T-1095 treatment improved SGLT1 to a level similar to the normal value. Therefore, administration of T-1095 may normalize the expression of SGLT1 in the small intestine by improvement of the hyperglycemia, and so probably does not have a harmful influence on the small intestine. GLUT2 occurs in the basolateral membrane of the renal tubule and intestinal basolateral membrane.[4,6] It has been reported that the expression of GLUT2 in primary hepatocytes is regulated by the plasma glucose level.[30] In this study, the expression levels of GLUT2 mRNA and protein in kidney and intestine were higher in STZ versus normal rats, and the elevated GLUT2 level was reduced by T-1095 to the level in normal rats. These results suggest that the improvement of hyperglycemia by T-1095 decreases the expression of renal and intestinal GLUT2.

We have demonstrated that plasma glucose, HbA_{1c}, urinary albumin, kidney weight, and vacuolation of epithelial cells in the tubules in insulin-deficient diabetic STZ rats can be decreased by oral administration of T-1095, and the expression of renal GLUT2 and intestinal SGLT1 and GLUT2 in T-1095-treated STZ rats are improved to the level of expressions in normal rats. T-1095, may therefore be an effective hypoglycemic agent in diabetes mellitus patients that also suppresses the progression of renal complications.

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