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Improved diabetic syndrome in C57BL/KsJ-db/db mice by oral administration of the Na^+-glucose cotransporter inhibitor T-1095

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1 The therapeutic effects of an orally active inhibitor of Na^+-glucose cotransporter (SGLT), T-1095 (a derivative of phlorizin; 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone 2′-*O*-(6-*O*-methoxycarbonyl-*β*-D-glycopyranoside)) were examined in C57BL/KsJ-db/db (db/db) mice, a genetic animal model of obese type 2 diabetes.

2 The higher renal SGLT activity in db/db mice than normoglycaemic C57BL/KsJ-db/+m (db/+m) mice may support the rationale for using an SGLT inhibitor in the treatment regimen for type 2 diabetes. Both T-1095 and its metabolite, T-1095A, which had approximately 10 times more potency, effectively inhibited renal SGLT activity of these mice *in vitro*.

3 Single oral administration of T-1095 (10, 30, 100 mg kg^{-1}, p.o.) to db/db mice caused a dose-dependent reduction in blood glucose levels and a concomitant increase in glucose excretion into urine. In contrast, T-1095 only slightly affected blood glucose levels in db/+m mice.

4 Chronic administration of T-1095 (0.1% w w^{-1} pellet chow, for 12 weeks) decreased blood glucose and haemoglobin A_{1C} levels, and improved glucose intolerance in db/db mice. The age-related decrease in plasma insulin levels was markedly inhibited and there was a 2.5 fold increase of insulin content in the pancreas of T-1095-treated db/db mice. Food consumption was not changed, while impaired body weight gain was ameliorated by T-1095 treatment.

5 Both the development of albuminuria and the expansion of glomerular mesangial area in db/db mice were significantly suppressed by chronic T-1095 treatment, indicating the prevention of the progression of diabetic nephropathy.

6 These results demonstrate that the SGLT inhibitor T-1095 is able to improve the metabolic abnormalities and inhibit the development of diabetic complications in db/db mice. Thus, T-1095 can be used for therapy of type 2 diabetic patients.

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Keywords: T-1095; Na^+-glucose cotransporter (SGLT); antidiabetic agents; diabetes; nephropathy; db/db mice

Abbreviations: AGE, advanced glycation end products; BBMV, brush border membrane vesicles, 95% CI, 95% confidence interval; db/db, C57BL/KsJ-db/db; db/+m, C57BL/KsJ-db/+m; DCCT, Diabetes Control and Complications Trial; DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunosorbent assay; GLUT4, glucose transporter subtype 4; HbA_{1C}, haemoglobin A_{1C}; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; IC_{50}, 50% inhibitory concentration; OGTT, oral glucose tolerance test; PAS, periodic acid Schiff; RIA, radioimmunoassay; SGLT, Na^+-glucose cotransporter; Tris, 2-amino-2-hydroxymethyl-propan-1,3-diol; UKPDS, U.K. Prospective Diabetes Study

Introduction

The major biochemical alteration in type 2 diabetes is hyperglycaemia, which is caused by variable combination of impaired insulin secretion from pancreatic *β*-cells and insulin resistance in peripheral tissues (DeFronzo *et al*., 1992; Taylor *et al*., 1994). Hyperglycaemia is not only a symptom of diabetes mellitus, but also a pathogenic factor leading to chronic diabetic micro- and macro-vascular complications (Klein, 1995; Porte & Schwartz, 1996). In addition, several lines of evidences suggest that hyperglycaemia *per se* directly impairs both insulin secretion and sensitivity, a phenomenon known as 'glucose toxicity', which contributes to the progressive worsening of hyperglycaemia (Rossetti, 1995). Thus, in type 2 diabetic patients, the goal of the therapy is to strictly control blood glucose levels within the normal range. However, due to limited efficacy and adverse side effects of currently available therapies, it is difficult to maintain good glycaemic control in most diabetic patients. Therefore, there is a strong incentive to develop new hypoglycaemic drugs.

Previous studies have suggested that the hypoglycaemic effect of phlorizin, a classic inhibitor of Na^+-glucose cotransporter (SGLT), is attributed glucosuria (Blondel *et al*., 1990; Khan & Efendic, 1995; Krook *et al*., 1997; Rossetti *et al*., 1990). Although phlorizin is ineffective when administered orally, presumably due to hydrolysis by *β*-glucosidase in the intestine (Malathi & Crane, 1969;

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Tsujihara *et al*., 1996), we have recently identified some orally active SGLT inhibitory compounds among analogues of phlorizin (Hongu *et al*., 1998a,b; Tsujihara *et al*., 1996). Based on its high potency and low toxicity, T-1095 (3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone 2′-*O*-(6-*O*-methoxycarbonyl-*β*-D-glycopyranoside) was selected from 4′-dehydrophlorizin derivatives for further pharmacological evaluations (Tsujihara *et al*., 1999). As reported previously, T-1095 showed hypoglycaemic effects in some diabetic animal models, streptozotocin-induced diabetic rats, yellow KK mice, and Zucker diabetic fatty rats (Nawano *et al*., 1999; 2000; Oku *et al*., 1999; 2000). It is therefore expected T-1095 is a potentially novel antidiabetic agent.

The purpose of the current study was to further characterize the actions of T-1095. We used the diabetic mouse strain C57BL/KsJ-db/db (db/db), which exhibits many of the metabolic disturbances of human type 2 diabetes including hyperglycaemia, obesity, and early hyperinsulinaemia (Berglund *et al*., 1978; Herberg & Coleman, 1977; Hummel *et al*., 1966). In addition, renal pathological changes after 10–20 weeks of sustained hyperglycaemia also resemble those observed in human diabetic patients (Cohen *et al*., 1994; Lee & Bressler, 1981). In the present study, the pharmacological effects of T-1095 with specific regard to blood glucose control and prevention of the progressive diabetic syndrome including diabetic nephropathy were evaluated in db/db mice.

Methods

Chemicals

T-1095 (3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone 2′-*O*-(6-*O*-methoxycarbonyl-*β*-D-glycopyranoside) and its metabolite T-1095A (3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone 2′-*O*-*β*-D-glycopyranoside) were synthesized at the Discovery Research Laboratory of Tanabe Seiyaku Co., Ltd. Phlorizin was purchased from Sigma (St. Louis, MO, U.S.A.). D-[6-^{3}H(N)]glucose was obtained from NEN (Boston, MA, U.S.A.). All other chemicals used were of guaranteed reagent grade.

Animals

Male db/db mice and their nondiabetic controls (db/+m) were obtained from CLEA Japan (Tokyo, Japan). They were housed individually in plastic cages with bedding and allowed free access to normal laboratory chow, CE-2 (CLEA Japan), and tap water. The animal rooms were controlled for temperature (23±2°C), humidity (55±5%), and light (12 h light-dark cycle). All mice were used for experiments at 8 weeks of age after 1 week of acclimation period. The animals were divided into experimental groups matched for both body weights and blood glucose levels. The ethics committee of Tanabe Seiyaku Co., Ltd approved all experimental procedures.

In vitro *inhibition of renal SGLT activity by T-1095 and T-1095A*

Brush border membrane vesicles (BBMV) were prepared from the renal cortex of db/+m and db/db mice by the Ca^{2+} precipitation method (Malathi *et al*., 1979). SGLT activity was assayed as Na^+-dependent [^{3}H]-glucose uptake in BBMV. In brief, BBMV suspension (0.2 mg protein) in 150 μl of assay buffer (10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES)-2-amino-2-hydroxymethyl-propan-1,3-diol (Tris), pH 7.4, 100 mM mannitol) was preincubated at 37°C for 2 min with or without compound. Compounds were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO was always kept at 0.5% and control BBMV also received equivalent amount of DMSO. The transport reaction was started by addition of 50 μl substrate (D-[6-^{3}H(N)] glucose 37 kBq; final concentration, 0.1 mM, and NaSCN or KSCN; final concentration, 100 mM) and stopped after 5 s by addition of 1.5 ml of ice-cold stop solution containing 150 mM NaCl and 0.3 mM phlorizin in 10 mM HEPES-Tris buffer (pH 7.4). BBMV were immediately filtered through a nitrocellulose membrane filter (pore size 0.45 μm; Advantec, Tokyo, Japan) under light suction and then washed with 4.5 ml of ice-cold stop solution. The radioactivity on the membrane filter was measured with a liquid scintillation counter (Tricarb 4640; Packard, Meriden, CT, U.S.A.).

The single administration study

For the single oral administration experiment, T-1095 suspended in 0.1% (w v^{-1}) hydrogenated caster oil polyethylene glycol ether (Nikkol® HCO-60; Nikko Chemicals, Tokyo, Japan) solution was orally administered to db/db and db/+m mice *via* a stomach tube at a volume of 10 ml kg^{-1}. Blood samples in the fed state were taken from the tail vein before and at 0.5, 1, 2, 3, 5, 8, and 24 h after the administration of the drug or vehicle for determination of glucose. Urine samples were collected using metabolic cages to measure urinary glucose excretion.

The chronic administration study

The db/db mice were kept on a CE-2 pellet chow containing 0.03 or 0.1% (w w^{-1}) of T-1095 for 12 weeks. The exact doses were estimated from the daily diet intakes and body weights. Blood samples in the fed state and 24 h urine samples were collected as described above. The levels of blood glucose, haemoglobin A_{1C} (HbA_{1C}), plasma insulin, urinary glucose and urinary albumin were determined periodically. An oral glucose tolerance test (OGTT) was performed at the 12th week of the study. At the end of the experimental period, the mice were killed by whole blood collection from the abdominal aorta under ether anaesthesia. Then, the kidneys and pancreas were removed quickly from each mouse and weighed. The pancreases were immediately frozen in liquid N_2, and were stored at −80°C for later measurement of insulin and glucagon contents. The kidneys were examined histopathologically as described below.

OGTT

Mice were fasted overnight and then 1 g kg^{-1} glucose solution was orally administered at a volume of 10 ml kg^{-1}. Blood samples were obtained before and 30, 60, and 120 min after the glucose challenge for determination of blood glucose levels.

Pancreatic insulin and glucagon contents

Pancreatic insulin and glucagon contents were determined after extraction by acid-ethanol solution. Whole pancreases were crushed and homogenized in acid-ethanol solution (75% EtOH, 23.5% d-water, 1.5% c-HCl) with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenized tissue was extracted overnight at 4°C, centrifuged at $1500 \times g$ for 30 min, and the resultant supernatant was diluted and then subjected to radioimmunoassay (RIA) for insulin and glucagon determinations.

Analytical methods

Blood glucose was determined using commercially available kits based on the glucose oxidase method (New Blood Sugar Test®; Boehringer Mannheim, Mannheim, Germany). Urinary glucose was measured by a Glucose Analyser (APEC, Inc., Danvers, MA, U.S.A.). HbA_{1C} was determined by an affinity column method (Glyc-Affin-GHb®; Seikagaku Corp., Tokyo, Japan). Plasma and pancreatic insulin contents were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Seikagaku Corp.) and a RIA kit (Amersham, Buckinghamshire, U.K.), respectively, with rat insulin as standards. Glucagon was measured with a RIA kit (Daiichi Radioisotope, Tokyo, Japan). Urinary albumin contents were determined using an ELISA kit (Exocell, Inc., Philadelphia, PA, U.S.A.) with mouse albumin as a standard.

Glomerular histology and morphometry

For histopathological examination, the kidneys were fixed in methanol-Carnoy's solution, and the specimens were embedded in paraffin. The sections (4 μm) were stained with the hematoxylin and eosin and periodic acid Schiff (PAS) techniques, and were examined under a light microscope. For quantification, sections were coded and read by an observer unaware of the experimental protocol applied. One hundred glomeruli (50 glomeruli each from left and right kidneys) were randomly selected from each animal. The extent of increase in mesangial area was determined by the presence of PAS-positive material in the mesangial region and scored as follows: 0, no remarkable change; 1, diffuse and slight increase; 2, segmental increase with nodular lesion; 3, global increase like a glomerulosclerosis. The total score of 100 glomeruli was used for the statistical analysis.

Statistics

Significant differences between groups were evaluated using unpaired Student's *t*-test or one-way analysis variance with multiple comparisons by Dunnett's method where appropriate. Histopathological score of glomerular lesions were analysed by the Kruskal-Wallis test followed by the Mann–Whitney *U*-test with Bonferroni's correction. In all cases, probabilities less than 5% ($P<0.05$) were considered to be statistically significant. Fifty per cent inhibitory concentration (IC_{50}) and 95% confidence interval (95% CI) were calculated by nonlinear least squares analysis using a four-parameter logistic model.

Results

Renal SGLT activity and in vitro *inhibition by T-1095 and T-1095A*

Renal SGLT activities of db/+m mice and db/db mice were determined at 8 weeks of age. Significantly higher activities were observed in db/db mice than in db/+m mice (191 ± 4 vs 148 ± 3 pmol s^{-1} mg $protein^{-1}$, mean ± s.e.mean of three separate membrane preparations each performed triplicate, $P<0.01$).

Figure 1 shows the effects of T-1095, T-1095A and phlorizin on the renal SGLT activities *in vitro*. All three compounds inhibited the activity in both db/+m (Figure 1a) and db/db mice (Figure 1b) in a concentration-dependent manner. The IC_{50} values (95% CI) of T-1095, T-1095A and phlorizin were 12.5 (10.7–14.6), 1.5 (1.1–2.1), and 2.9 (2.0–4.0) μM in db/+m mice, and 12.4 (10.5–14.7), 1.1 (0.9–1.5) and 2.5 (2.0–3.2) μM in db/db mice, respectively. There was no difference in the inhibitory potencies of these compounds between db/+m and db/db mice. In terms of IC_{50} values, T-

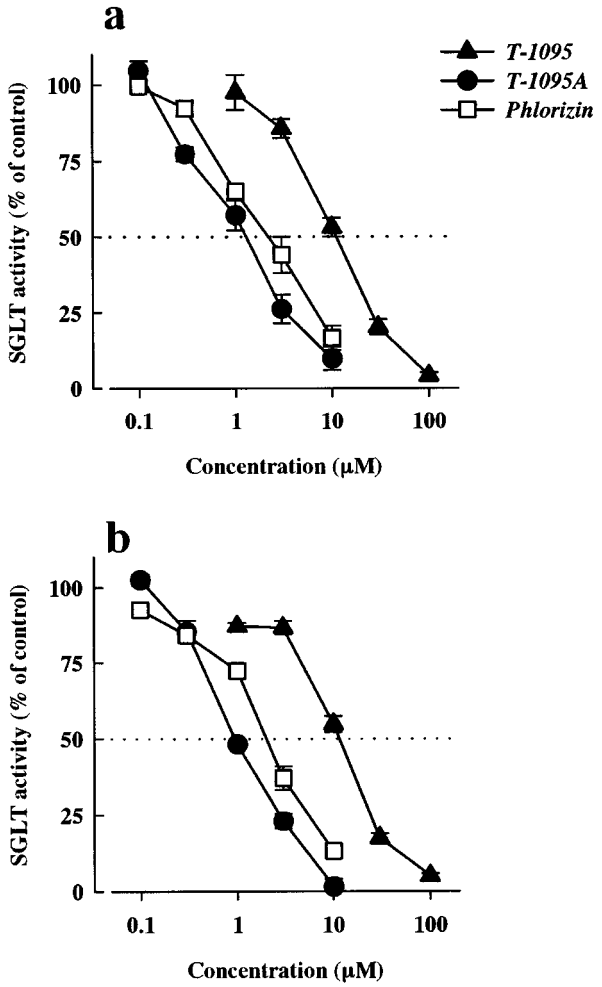

Figure 1 Effect of T-1095, T-1095A and phlorizin on renal SGLT activity of db/+m (a) and db/db mice (b) *in vitro*. SGLT activity was assayed as Na^+-dependent [^{3}H]-glucose uptake (0.1 mM) in BBMV prepared from the renal cortex. Symbols represent mean values and vertical lines show s.e.mean of three observations.

1095A was approximately two and 10 times more potent than phlorizin and T-1095, respectively.

Effect of single oral administration of T-1095 on blood glucose and urinary glucose excretion

When 10, 30, and 100 mg kg^{-1} of T-1095 were orally administered to db/db mice, dose-dependent blood glucose lowering effects were observed (Figure 2b). A significant fall of blood glucose levels could be detected at a dose of 30 mg kg^{-1}. The maximal dose of 100 mg kg^{-1} caused sustained decrease in blood glucose levels for 5 h, but a significant reduction was no longer noticed after 8 h. In contrast to db/db mice, the effect of T-1095 on blood glucose levels in normoglycaemic db/+m mice was only marginal even at 100 mg kg^{-1} (Figure 2a).

Table 1 shows the urinary glucose excretion of the experimental groups. The glucosuria of db/db mice were dose-dependently accelerated by T-1095 administration in 5 h (0–5 h), although no changes were detected thereafter (5–24 h). In db/+m mice, there was a dose-dependent increase in cumulative urinary glucose (0–24 h) after oral administration of T-1095. The increase of urinary glucose excretion was more pronounced in 5 h after T-1095 administration in db/db mice than that in 24 h in db/+m mice.

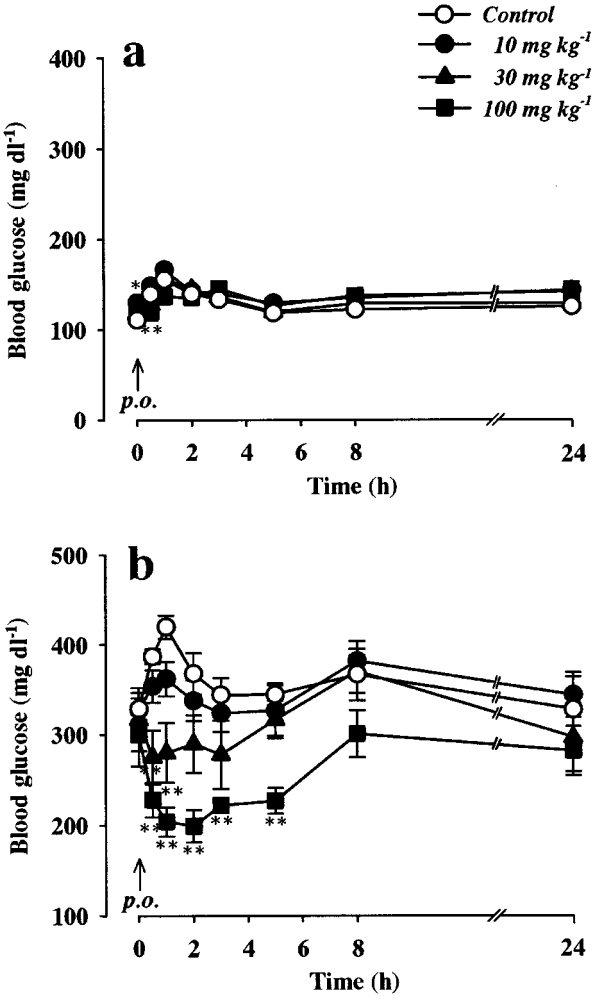

Figure 2 Effect of single oral administration of T-1095 on blood glucose levels in db/+m (a) and db/db mice (b). T-1095 (10, 30 and 100 mg kg^{-1}) was orally administered and changes in blood glucose were monitored for 24 h. Symbols represent mean values and vertical lines show s.e.mean ($n=5$). $^{*}P<0.05$, $^{**}P<0.01$ *versus* respective control.

Effect of chronic administration of T-1095 on the glycaemic control and the progressive diabetic phenotype

The db/db mice were kept on a diet containing 0.03 (low dose) or 0.1% (high dose) (w w^{-1}) of T-1095 for 12 weeks. The average daily dose of the drugs calculated from periodically determined food intake and body weight were as follows; T-1095 0.03%, 50 mg kg^{-1} day^{-1}; T-1095 0.1%, 152 mg kg^{-1} day^{-1}. Throughout the experimental period, T-1095 dose-dependently lowered blood glucose levels (Figure 3a) and HbA_{1C} values (Figure 3b) in db/db mice. Figure 4 shows the results of the OGTT performed at the 12th week of the treatments. T-1095 at the high dose improved the severe glucose intolerance of db/db mice; blood glucose levels in fasting and after the glucose load were both significantly lower in the mice than the untreated db/db mice.

The age-related changes of plasma insulin levels are shown in Figure 3c. The elevated plasma insulin levels in db/db mice at the beginning of the study were gradually decreased to values near those in db/+m mice with ageing. However, T-1095 at a high dose almost completely inhibited the age-related decrease of plasma insulin levels in these mice. The pancreatic insulin and glucagon contents and pancreatic tissue weights were also measured at the end of the study (Table 2). The pancreatic insulin content was markedly lower, whereas the glucagon content was higher in db/db mice than db/+m mice. The T-1095 treatment at a high dose resulted in significant increase (approximately 2.5 fold) of pancreatic insulin contents, but not the pancreatic glucagon contents and pancreatic tissue weights.

The effect of the treatments on body weight of db/db mice is shown in Figure 3d. Body weights of T-1095-treated db/db mice were comparable with control db/db mice up to 10 weeks of age. The control db/db mice did not gain more weight from this age, while T-1095-treated db/db mice further gained weight dose-dependently. Interestingly, as shown in Table 2, T-1095 treatment did not affect the food consumption throughout the study. In addition, T-1095 treatment also ameliorated polydipsia (Table 2), polyuria (Figure 5a), and glucosuria (Figure 5b) of db/db mice.

As shown in Figure 5c, urinary microalbumin excretion, the established parameter reflecting diabetic glomerular dysfunction (Mogensen, 1990), was significantly increased in db/db mice at the start of the experiment. Then, the urinary albumin was gradually increased, and reached approximately 1.2 mg 100 g^{-1} day^{-1}, which was as 30 times the level as that of db/+m mice. T-1095 at high dose almost completely suppressed the increase of urinary albumin, indicating the beneficial influence on renal dysfunction in these mice. The protective effect of T-1095 on nephropathy was also examined by the histopathological analyses. Renal glomeruli in db/db controls showed various extent of expansion of the PAS-positive mesangial area (Figure 6b) in contrast to db/+m mice (Figure 6a). Glomeruli from T-1095-treated db/db mice were with appreciably less mesangial expansion (Figure 6c). Scoring the extent of increase in mesangial area

Table 1 Effects of single oral administration of T-1095 on urinary glucose excretion in db/+m and db/db mice

Groups	*Dose* (mg kg^{-1})	*Cumulative urinary glucose excretion* (mg 100 g^{-1}) *db/+m 0–24 h*	*db/db 0–5 h*	*db/db 5–24 h*
Control		5±1	154±44	1691±142
T-1095	10	3±0	234±52	2085±216
	30	57±24	334±91	1435±404
	100	169±29**	447±68*	1563±347

T-1095 (10, 30 and 100 mg kg^{-1}) was orally administered and 24 h-urine samples (from 0 to 24 h for db/+m mice; from 0 to 5 h and from 5 to 24 h for db/db mice) were collected using metabolic cages. Values are mean±s.e.mean ($n=5$). *$P<0.05$ and **$P<0.01$ *versus* respective control.

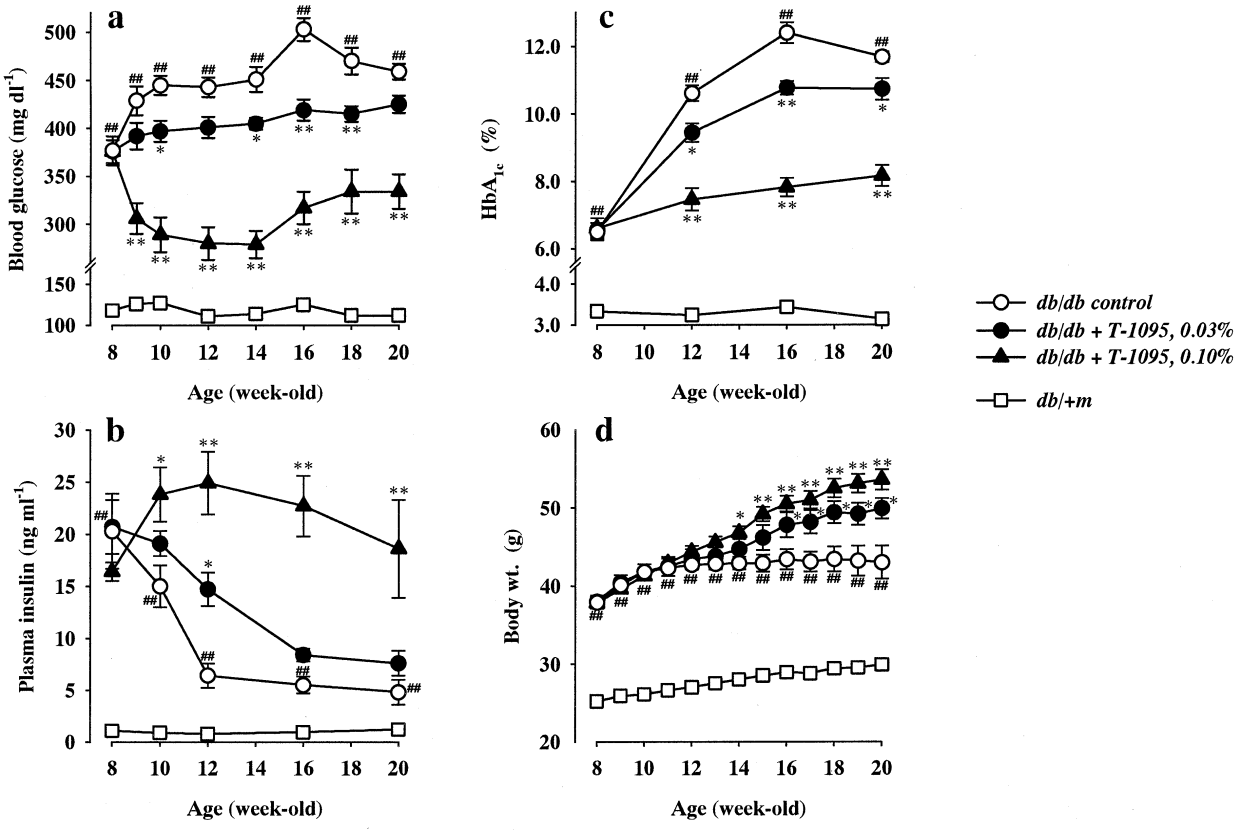

Figure 3 Effects of chronic T-1095 treatment on blood glucose (a), HbA_{1C} (b), plasma insulin (c) levels and body wt. (d) in db/db mice. T-1095 (0.03 and 0.10% in diet) was given for 12 weeks. Blood glucose, HbA_{1C}, plasma insulin levels and body wt. were monitored periodically. Symbols represent mean values and vertical lines show s.e.mean ($n=8$). $^{\#\#}P<0.01$ *versus* db/+m mice. *$P<0.05$, **$P<0.01$ *versus* control.

quantitates these differences; 65±4, 56±4, 46±4, and 4±1 in untreated db/db, low dose T-1095, high dose T-1095, and db/+m groups, respectively. T-1095 at a high dose significantly ($P<0.01$) decreased the mesangial expansion. The low dose T-1095 group also exhibited tendency to decrease mesangial area, whereas the difference did not reach significance.

Discussion

Plasma glucose is filtered in the glomerulus and then transepithelially reabsorbed by SGLT in the proximal tubules (Deetjen *et al*., 1995; Silverman & Turner, 1992). The reabsorption of glucose is saturable, and when the amount of the filtered glucose exceeds the capacity due to hyperglycaemia, non-reabsorbed glucose is excreted in urine, i.e. glucosuria (Deetjen *et al*., 1995). The significant higher renal SGLT activity in db/db mice suggests enhanced glucose reabsorption in diabetic mice than nondiabetic mice. In diabetic patients, glucosuria can be considered as a protective overflow, since more severe hyperglycaemia is prevented (Deetjen *et al*., 1995). Thus, although it may not be a primary factor for induction of diabetes, elevated renal glucose reabsorption would further aggravate hyperglycaemia. This may support the rationale for using an SGLT inhibitor as a

hypoglycaemic agent in the treatment regimen for diabetes mellitus.

Bolus p.o. administration of T-1095 caused dose-dependent falls in the blood glucose levels in db/db mice. Since there was an additional excretion of glucose into urine, blood glucose lowering by T-1095 is likely a consequence of glucosuria induction *via* the inhibition of renal glucose reabsorption. In contrast to db/db mice, there was only a marginal hypoglycaemic effect and urinary glucose increase by T-1095 administration in normoglycaemic db/+m mice. As mentioned above, since SGLTs are abundantly expressed in proximal tubules, only a part of the SGLT function is sufficient for complete reabsorption of filtered glucose at normal glucose levels; in turn, the reabsorption mechanism is saturated under hyperglycaemic conditions (Deetjen *et al*., 1995). Some extent of inhibition of SGLT would excrete larger amount of glucose into urine in hyperglycaemic conditions than in normoglycaemic state and, therefore, T-1095 lowers the blood glucose levels more efficiently in hyperglycaemic than normoglycaemic states. This can be considered as an advantage, because T-1095 hardly induces hypoglycaemia, which is a major concern for current therapies with insulin or sulphonylureas.

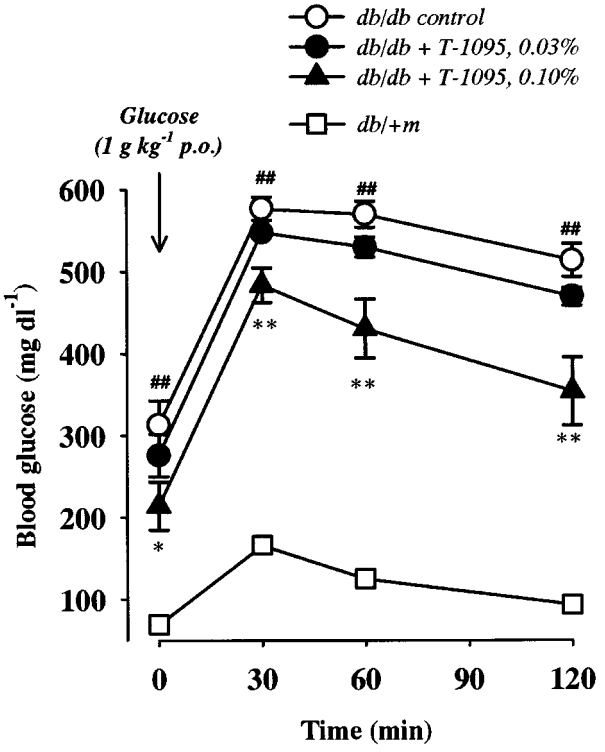

Figure 4 Effects of chronic T-1095 treatment on glucose intolerance in db/db mice. T-1095 (0.03 and 0.10% in diet) was given for 12 weeks. Mice were subjected to an OGTT after an overnight fast. Following baseline blood glucose determination, glucose (1 g kg^{-1} body wt.) was orally administered and blood glucose levels were evaluated through 120 min. Symbols represent mean values and vertical lines show s.e.mean ($n=8$). $^{\#\#}P<0.01$ *versus* db/+m mice. $^{*}P<0.05$, $^{**}P<0.01$ *versus* control.

On the other hand, SGLT is also abundantly expressed in small intestine and mediates intestinal glucose absorption (Silverman, 1991). Therefore, it cannot be ruled out the possibility that suppression of the intestinal glucose absorption also contribute, at least in part, to the anti-hyperglycaemic action of T-1095. A preliminary study has shown that T-1095 is converted to T-1095A by S9 fraction of the liver and that T-1095A but not T-1095 was detected in plasma following oral administration of T-1095 (unpublished observations). Both T-1095 and T-1095A inhibited renal SGLT, but in terms of IC_{50} values, the former agent is approximately 10 times less potent than the later. Thus, it is likely that T-1095A primarily accounts for the hypoglycaemic effects of T-1095 in db/db mice *via* renal SGLT inhibition.

This study has also shown that chronic treatment of db/db mice with T-1095 improves glycaemic control and ameliorates severe glucose intolerance. Interestingly, T-1095 did not increase but rather decreased the urinary glucose excretion in the experiment. In addition, both the urine volume and water intake in db/db mice were reduced, probably due to the decrease of urinary glucose excretion. These results are apparently paradoxical, but reasonable, because the glucose content in glomerular filtrate, which is a linear function of the blood glucose levels, would decrease in T-1095-treated mice along with the correction of hyperglycaemia. However, when the blood glucose levels of db/db mice were controlled

Table 2 Effects of chronic administration of T-1095 on food consumption, water consumption, kidneys wt., pancreas wt., pancreatic insulin and glucagon contents in db/db mice

	Age (weeks)	*db/db Control*	*db/db + T-1095 0.03%*	*db/db + T-1095 0.10%*	*db/+m*
Food consumption (g day^{-1})	9	$7.2\pm0.1^{\#\#}$	7.8 ± 0.3	7.3 ± 0.4	3.4 ± 0.1
	12	$7.5\pm0.2^{\#\#}$	7.3 ± 0.3	7.4 ± 0.3	3.8 ± 0.1
	16	$7.0\pm0.2^{\#\#}$	6.6 ± 0.3	6.3 ± 0.4	3.4 ± 0.1
	20	$6.3\pm0.3^{\#\#}$	7.2 ± 0.2	6.9 ± 0.3	3.7 ± 0.1
Water consumption (ml day^{-1})	9	$21.6\pm1.6^{\#\#}$	19.8 ± 1.3	16.8 ± 1.9	5.1 ± 0.1
	12	$24.6\pm1.1^{\#\#}$	20.6 ± 1.3	$17.6\pm1.7^{**}$	4.8 ± 0.1
	16	$22.2\pm1.5^{\#\#}$	$16.9\pm1.4^{*}$	$12.9\pm1.3^{**}$	4.1 ± 0.2
	20	$18.8\pm1.8^{\#\#}$	20.6 ± 1.8	16.6 ± 1.9	4.2 ± 0.2
Kidneys wt. (mg)	20	$552\pm25^{\#\#}$	550 ± 25	555 ± 13	470 ± 12
Pancreas wt. (mg)	20	314 ± 10	344 ± 22	364 ± 13	318 ± 13
Pancreatic insulin (ng mg^{-1})	20	$32.9\pm4.0^{\#\#}$	49.3 ± 6.8	$80.7\pm11.3^{**}$	245.0 ± 16.4
Pancreatic glucagon ng mg^{-1})	20	$23.0\pm1.7^{\#\#}$	20.4 ± 2.5	18.1 ± 2.7	6.3 ± 0.8

Drugs were given as dietary admixtures for 12 weeks. Food and water intakes were monitored periodically. Kidneys and pancreas wt., pancreatic insulin and glucagon contents were determined at the end of the study. Values are means±s.e.mean ($n=8$). $^{\#\#}P<0.01$ *versus* db/+m mice. $^{*}P<0.05$ and $^{**}P<0.01$ *versus* db/db control.

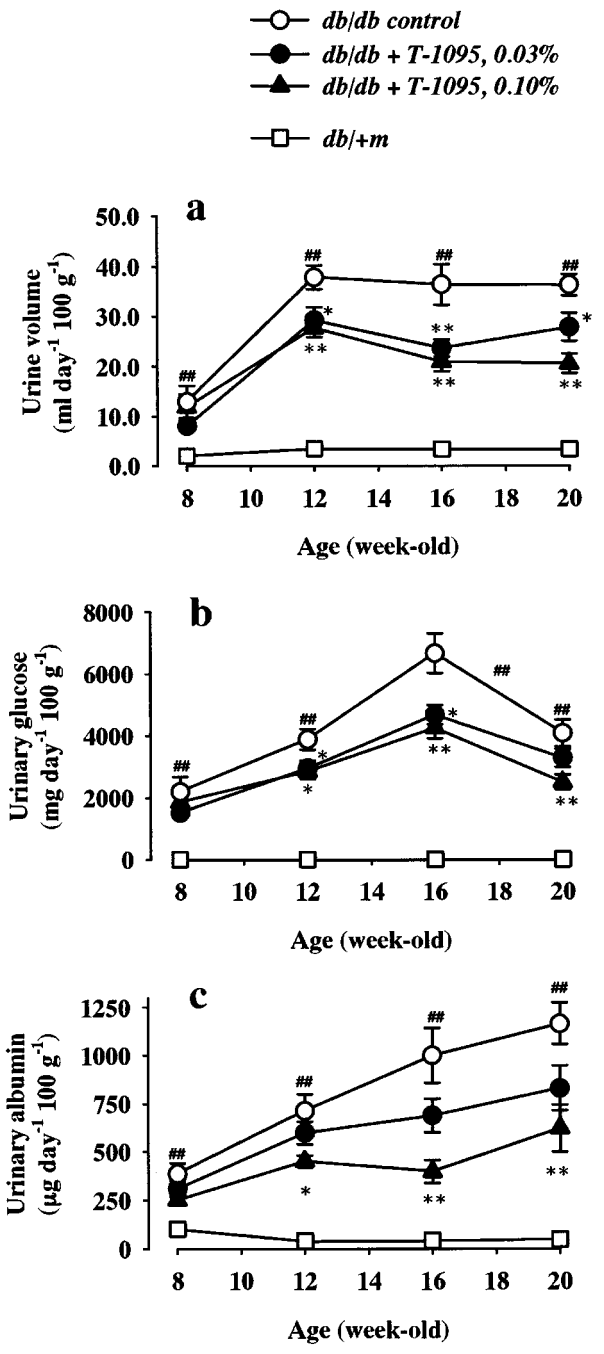

Figure 5 Effect of chronic T-1095 treatment on urine volume (a), urinary glucose (b) and albumin (c) excretion in db/db mice. T-1095 (0.03 and 0.10% in diet) was given for 12 weeks. Urine was collected using metabolic cages and urine volume, urinary glucose and albumin excretion were monitored periodically. Symbols represent mean values and vertical lines show s.e.mean ($n=8$). $^{\#\#}P<0.01$ *versus* db/+m mice. $^{*}P<0.05$, $^{**}P<0.01$ *versus* control.

similarly with acarbose, an α-glucosidase inhibitor, there was much less urinary glucose excretion compared with animals treated with T-1095 (data not shown). Therefore, it is likely that T-1095 inhibits reabsorption of glucose and still excretes more glucose even when hyperglycaemia is continuously suppressed, and maintains its blood glucose-lowering action.

As there was no decrease in the food consumption of T-1095-treated-db/db mice, the continuous hypoglycaemic effect is not due to decrease in caloric intake. T-1095-treated db/db mice gained weight throughout the study, although the control db/db mice did not gain more weight from 10 weeks of age. Similar improved weight gain in db/db mice has also been reported in troglitazone-treated (Fujiwara *et al*., 1991) and glucose transporter subtype 4 form (GLUT4) over-expressed db/db mice (Gibbs *et al*., 1995). Since db/db mice do not add weight along with progression of diabetic phenotype, we hypothesize that improvement of hyperglycaemia by T-1095 prevents the development of diabetic state and thus enhances the animals' ability to thrive.

Although glucose is the major physiological stimulator of insulin secretion and biosynthesis, extensive exposure of pancreatic β cells to high levels of glucose *in vitro* causes β-cell dysfunction that is associated with impaired insulin secretion and biosynthesis (Robertson *et al*., 1992). There is substantial evidence linking hyperglycaemia with the non-enzymatic reaction of sugars with proteins and the accelerated formation of advanced glycation end products (AGE) that may play an important role in the pathogenesis of glucose toxicity (Tajiri *et al*., 1997). Prolonged poor glycaemic control in diabetic patients often leads to a decline in insulin secretion from pancreatic β cells and to worsening of the diabetic state (Unger & Grundy, 1985), as is in the case of db/db mice. In the present study, we demonstrated that the long-term treatment with T-1095 prevented the age-related decrease in plasma insulin levels and attenuated the loss of pancreatic insulin contents in db/db mice. As T-1095 showed little effect on blood glucose levels in db/+m mice, it is unlikely that it has direct insulinotrophic actions on pancreatic β cells. This is also supported by the hypoglycaemic effects of T-1095 in insulin-deficient diabetic rats (Oku *et al*., 1999). Recent study has shown that similar beneficial effects of aminoguanidine, an inhibitor of AGE formation, on serum and pancreatic insulin levels of db/db mice (Piercy *et al*., 1998). It is likely that these drugs prevented exhaustion of pancreatic β cells of db/db mice possibly through the alleviation of glucose toxicity.

Diabetic nephropathy is the single largest cause of end-stage renal disease and develops in many patients with both type 1 and type 2 diabetes. Effective medical therapy to halt the inexorable progression of renal disease due to diabetes, once the process is initiated, has been elusive. For this reason, we also studied the effect of T-1095 on diabetic nephropathy in db/db mice. Albumin excretion, the established parameter reflecting the diabetic glomerular dysfunction (Mogensen, 1990), was age-dependently increased in db/db mice. Furthermore, the morphological study has demonstrated an expansion of glomerular mesangial area like human diabetic nephropathy in aged db/db mice. Marked improvement of these parameters suggests that the treatment with T-1095 prevents the development of renal dysfunction in the diabetic mice. The results of the Diabetes Control and Complications Trial (DCCT) (DCCT, 1995) and U.K. Prospective Diabetes Study (UKPDS) (UKPDS, 1998) have established that those intensive regimens for blood glucose control lower the risk for diabetic nephropathy. Therefore, it is likely that T-1095 prevents the development of diabetic nephropathy by reducing blood glucose levels at an early stage of diabetes. Suppression by acarbose of the nephropathy of db/db mice

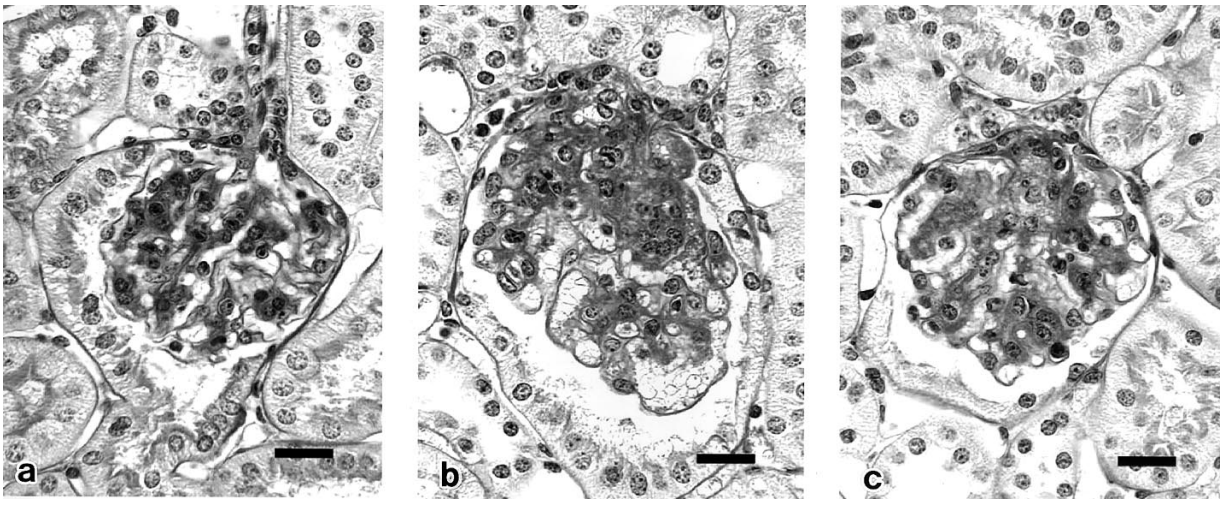

Figure 6 Representative microphotographs of glomeruli from db/+m mice (a), control db/db mice (b), and the high dose of T-1095-treated db/db mice (c). Expansion of the PAS-positive glomerular mesangial area is apparent in the control db/db mice compared with the db/+m mice. There is minimal mesangial widening in the T-1095-treated mice. Scale bar = 10 μm.

(Lee, 1982), also supports this conclusion. However, a recent report suggested the involvement of the augments glucose influx into mesangial cells in development of diabetic nephropathy (Heilig *et al*., 1997). Although the physiological role of SGLT in mesangial cells is not known, the direct renal protective action *via* an effect on the mesangial SGLT cannot be excluded, and remains to be investigated.

In summary, we demonstrated the acute and chronic antihyperglycaemic effects of T-1095 in db/db mice. In addition, long-term treatment with T-1095 restored the deterioration of diabetic states, possibly through protection from glucose toxicity, and suppressed the development of diabetic nephropathy. Thus it is expected that an orally active SGLT inhibitor, T-1095, can be used for the therapy of human type 2 diabetic patients.

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