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Antidiabetic effect of T-1095, an inhibitor of Na^+-glucose cotransporter, in neonatally streptozotocin-treated rats

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Abstract

3-(Benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-(6-*O*-methoxycarbonyl)-β-D-glucopyranoside (T-1095) is a derivative of phlorizin, a potent inhibitor of Na^+-glucose cotransporters. We determined the antidiabetic effect of T-1095 in neonatally streptozotocin-treated diabetic rats. Orally administered T-1095 is metabolized to an active form, 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-β-D-glucopyranoside (T-1095A), which inhibits renal Na^+-glucose cotransporters as potently as phlorizin in vitro. A single oral administration of T-1095 (30 and 100 mg/kg, p.o.) markedly lowered blood glucose levels with a concomitant increase in urinary glucose excretion; whereas the effect on blood glucose levels in non-diabetic rats was minimal. Continuous administration of T-1095 to diabetic rats for 6 weeks (0.1% in diet) improved not only hyperglycemia, but also the elevation of plasma free fatty acid and plasma ketone body levels. In addition, oral glucose tolerance testing clearly illustrated the improvement of glucose tolerance and insulin secretion with T-1095. In fact, amelioration of impaired insulin sensitivity in diabetic rats was demonstrated by the increase of whole-body and skeletal-muscle insulin-mediated glucose utilization with normalization of muscle glucose transporter (GLUT)4 content, and decrease of the hepatic glucose production rate. Consequently, polyuria and glucosuria were also improved in the T-1095-treated group. Therefore, T-1095 has a therapeutic potential as a means of ameliorating abnormal glucose metabolism via diminished glucose toxicity.

Keywords: Streptozotocin; T-1095; Phlorizin; Na^+-glucose cotransporter; Glucose clamp; Glucose tolerance test; Glucose transporter; Insulin sensitivity; Insulin secretion

1. Introduction

Defects in insulin secretion and insulin action are universally present in type 1 diabetes and also in most type 2 diabetes in both human patients and animal models, although it has become increasingly clear that human diabetes is a heterogeneous disorder (Rossetti et al., 1990). When the hyperglycemia becomes of sufficient magnitude to lead to glucosuria and polyuria, a vicious cycle develops in which hyperglycemia leads to the worsening of insulin secretion by β cells and impairment of insulin sensitivity in peripheral tissues, resulting in more hyperglycemia (Rossetti et al., 1990). Thus, there are complex inter-relationships among hyperglycemia, insulinopenia, and insulin resistance, all of which are involved in the pathogenesis of diabetes (Rossetti et al., 1990). To date, several oral drugs and insulin have been developed for the treatment of diabetes (Lebovitz, 1994). However, it is still difficult to maintain good glycemic control in most diabetic patients, although individual drugs may be highly effective for some patients (Lebovitz, 1994).

We have developed a novel agent, 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-(6-*O*-methoxycarbonyl)-β-D-glucopyranoside (T-1095) (Tsujihara et al., 1999), which may be useful for the treatment of diabetic patients. T-1095 is a synthetic phlorizin derivative, which inhibits renal Na^+-glucose cotransporter activ-

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ity when administered orally (Oku et al., 1999). After being absorbed from the gut into the blood, T-1095 is metabolized to 3-(benzo[*b*]furan-5-yl)-2′,6′-dihydroxy-4′-methylpropiophenone-2′-*O*-β-D-glucopyranoside (T-1095A) and inhibits specifically and competitively Na^+-glucose cotransporters (Oku et al., 1999), which are responsible for glucose reabsorption in the proximal tubules (Silverman and Turner, 1992; Deetjen et al., 1995). Na^+-glucose cotransporter also mediates glucose absorption in the small intestine, but our previous study suggests that the effect of T-1095 on intestinal glucose absorption is negligible, and that the antihyperglycemic effect of T-1095 is primarily mediated by reduced renal glucose reabsorption (Oku et al., 1999). Thus, T-1095 lowers blood glucose by a novel mechanism for an antidiabetic drug.

In the present study, T-1095 was administered orally to diabetic rats that had been treated with streptozotocin as neonates. We now describe the alterations in various parameters associated with diabetic status as well as effects on the insulin secretory capacity and insulin sensitivity of the diabetic rats. Then, we discuss the potential application of T-1095 as a novel antidiabetic drug.

2. Materials and methods

Experiments were performed in accordance with the National Institute of Health ''Principles of Laboratory Animal Care, 1985 revised version,'' and had the approval of the ethics committee of Tanabe Seiyaku.

2.1. Chemicals

T-1095 (Fig. 1a), and its metabolite, T-1095A (Fig. 1b) were synthesized at the Discovery Research Laboratory in Tanabe Seiyaku. All other chemicals were standard high-purity materials obtained from commercial sources.

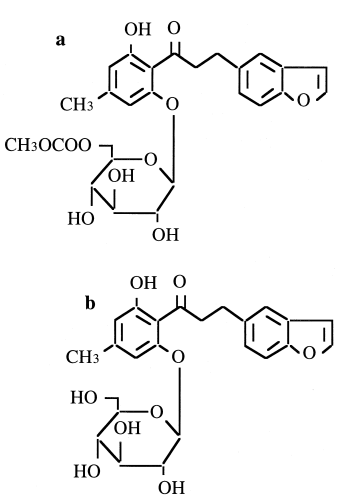

Fig. 1. Chemical structure of T-1095 (a) and its metabolite, T-1095A (b).

2.2. Animals

Male Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) were injected with streptozotocin (Sigma, St. Louis, MO, USA) intraperitoneally (60 mg/kg in 50 mmol/l citrate buffer, pH 4.5) 6 days after birth (diabetic rats). Normal (non-diabetic) groups received the buffer only. All animals were weaned 21 days after birth and were housed in stainless wire cages and given normal laboratory chow (CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum. The animals were used for the experiments described below at 6 weeks of age (body weight: normal, 164.8 ± 2.8 g, diabetic, 158.6 ± 1.7 g, means ± SEM, $n = 45$). A summary of the experimental procedure is shown in Fig. 2.

2.3. Inhibition of Na^+-glucose cotransporter activity in brush border membrane vesicles

Brush border membrane vesicles were prepared from renal tissues of normal and diabetic rats by the Ca^+ precipitation method (Malathi et al., 1979). Na^+-glucose cotransporter activity was determined by the rapid filtration method (Malathi et al., 1979). In brief, 50 μl of brush border membrane vesicles (100 μg membrane protein) in assay buffer (10 mmol/l HEPES–Tris, pH 7.4, 100 mmol/l mannitol) was preincubated at 37°C for 2 min, and combined with the test compounds, D-glucose (final 0.1 mmol/l), D-[6-^{3}H(N)]glucose (1 μCi, NEN, Boston, MA, USA), and NaSCN or KSCN (final 100 mmol/l) in 150 μl of assay buffer. Five seconds later, the uptake reaction was terminated by the addition of 1.5 ml of ice-cold stop solution containing 150 mmol/l NaCl and 0.3 mmol/l phlorizin in 10 mmol/l HEPES–Tris (pH 7.4). The vesicles were immediately filtered through a nitrocellulose membrane filter (pore size, 0.45 μm; Advantec Toyo, Japan) under light suction, and then washed with 4.5 ml of ice-cold stop solution. The radioactivity on the membrane was measured with a liquid scintillation counter (Tricarb 4640, Packard, Meriden, CT, USA).

2.4. Single administration of T-1095

At 6 weeks of age, the animals were divided into experimental groups matched for both body weights and blood glucose levels. T-1095 suspended in 0.1% (w/v) hydrogenated castor oil polyethylene glycol ether (Nikkol® HCO-60, Nikko, Japan) solution was administered orally to normal and diabetic rats via a stomach tube in a volume of 5 ml/kg. Blood samples for determination of glucose levels were taken from the tail vein before and at 1, 2, 3, 5, 8, and 24 h after administration of the drug or vehicle. Urine samples were collected using metabolic cages.

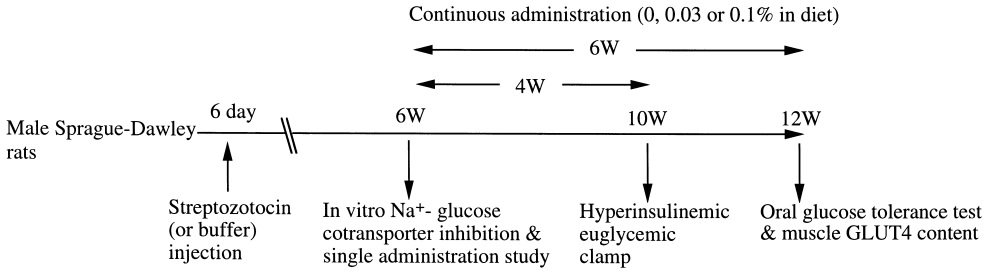

Fig. 2. Summary of experimental procedures.

2.5. Continuous administration of T-1095

In the continuous administration study, T-1095 was given as food admixtures. Both normal and diabetic rats (6 weeks old) were kept on a CE-2 diet containing 0% (control diet), 0.03%, or 0.1% (w/w) T-1095. The doses were estimated from the food intake and body weights. Blood and urine samples were collected once a week, as described above.

2.6. Glucose tolerance test

An oral glucose tolerance test was performed after 6 weeks on the various diets. Rats were fasted overnight and then a 1-g/kg glucose solution was administered orally. Blood samples were obtained before and 15, 30, 60, 120, and 180 min after the glucose challenge for determination of blood glucose and plasma insulin levels. Urine samples were collected during the test, as described above.

2.7. Hyperinsulinemic euglycemic clamp

After 4 weeks on the diet, the rats were fasted overnight. Under sodium thiobutabarbital (100 mg/kg, i.p.) anesthesia, tracheotomy was performed to avoid respiratory problems during anesthesia, and the urinary bladder was catheterized to collect urine. An intravenous catheter filled with heparin-saline (50 U/ml) was inserted into one of the jugular veins (for blood sampling and tracer injection). Two other catheters were inserted into the right and left femoral veins. One of these was used to infuse insulin and [^{14}C]glucose, the other to infuse a 10% (w/v) glucose solution.

A 3.2 $U \cdot kg^{-1} \cdot h^{-1}$ dose of human insulin (Humulin® R, Eli Lilly, Indianapolis, IN, USA) was infused over 2 h. The plasma glucose concentration was monitored every 5 min and clamped at 100–110 mg/dl by adjusting the rate of infusion of the 10% glucose solution delivered via the femoral cannula. The glucose infusion rate during the second hour of clamping was taken as the response parameter for whole-body insulin action. Blood samples (200 μl) were obtained for insulin determination in all studies at 60, 80, 100, and 120 min. Urine used for glucose determination was collected during the second hour via the urinary bladder cannula.

2.8. In vivo glucose utilization and glucose production

Forty minutes after commencement of the study, D-[U-^{14}C]glucose (Amersham Life Science, Buckinghamshire, England) was administered as an initial intravenous priming dose (4 μCi), immediately followed by continuous

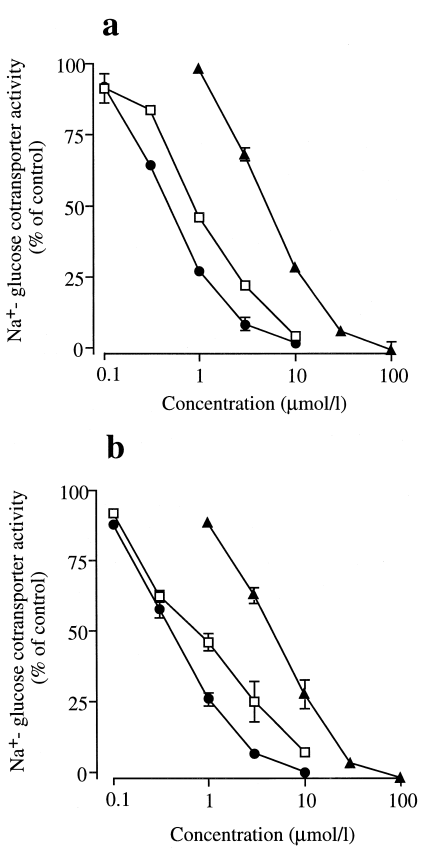

Fig. 3. Effects of T-1095, T-1095A, and phlorizin on Na^+-glucose cotransporter activity in renal brush border membrane vesicles prepared from normal (a) and diabetic (b) rats. ▲ T-1095; ● T-1095A; □ phlorizin. Na^+-glucose cotransporter activity was measured in the presence of 0.1 mmol/l D-[^{3}H]glucose and various concentrations of three drugs at 37°C by the rapid filtration method as described in Section 2. Values are means ± SEM (triplicate).

infusion at a rate of 0.2 μCi/min. The non-metabolizable glucose analog, 2-deoxy-[1-^{3}H]glucose (50 μCi, Amersham), was administered as an intravenous bolus at 75 min. Blood samples for the determination of plasma tracer concentrations (200 μl) were collected at 2, 5, 10, 15, 20, 30, and 45 min after bolus administration of 2-deoxy-[1-^{3}H]glucose. On completion of the clamp experiment, liver, skeletal muscle (quadriceps femoris), white adipose tissue (epididymal fat), and brown adipose tissue (interscapular fat) were rapidly removed and frozen using aluminum tongs precooled in liquid nitrogen. Twenty to 200 mg samples of tissues were weighed, dissolved in Soluen® 350 (Packard), then ^{3}H activity was measured. The rate of glucose disappearance (R_d) was calculated by dividing the [^{14}C]glucose infusion rate ($dpm \cdot min^{-1} \cdot kg^{-1}$ body weight) by the steady-state value of glucose specific activity (dpm/mg). The glucose utilization rate (GUR) based on whole-body mass and the glucose production rate (GPR) by the liver were calculated as follows: GUR = R_d − [urinary glucose loss]; and GPR = R_d − [steady-state glucose infusion rate]. An estimate of tissue glucose uptake (defined as the glucose utilization index, R'_g) was calculated as described by James et al. (1986).

2.9. GLUT1 and GLUT4 contents in skeletal muscles

After the 6-week T-1095 treatment, the rats were killed by exsanguination via the abdominal aorta under sodium pentobarbital anesthesia (50 mg/kg, i.p.) and skeletal muscles from hindlimbs were rapidly removed, then frozen using aluminum tongs precooled in liquid nitrogen. Samples were stored at −80°C until use for membrane preparation. Membranes of skeletal muscles were prepared as described by Dimitrakoudis et al. (1992a,b). Briefly, 8–12 g of the tissue was minced in buffer A (250 mmol/l sucrose, 5 mmol/l sodium azide, 0.1 mmol/l phenylmethyl sulfonyl fluoride, 10 mmol/l $NaHCO_3$ [pH 7.0]) and homogenized with a Polytron (Brinkman Instruments, Westbury, NJ, USA). This homogenate was centrifuged at $1200 \times g$ for 10 min and the pellet was re-homogenized and re-centrifuged to remove debris. The combined supernatants were centrifuged at $9000 \times g$ for 10 min, and the resulting supernatant was centrifuged at $190{,}000 \times g$ for 60 min to obtain a crude membrane preparation. The membrane preparation was suspended in buffer A, and the protein concentration was assayed with BCA® protein assay reagent (Pierce, Rockford, IL, USA). Twenty micrograms of membrane protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% (w/v) polyacrylamide) and electrophoretically transferred to polyvinylidene difluoride membranes at 45 V (constant) overnight. Immunoblotting with anti-GLUT1 and anti-GLUT4 antibodies (Katagiri et al., 1996) was performed with enhanced chemiluminescence (ECL®, Amersham), and the intensities of signals were quantified with a photoimager system (Quantity One Ver. 3.0.2, PDI, Huntington Station, NY, USA).

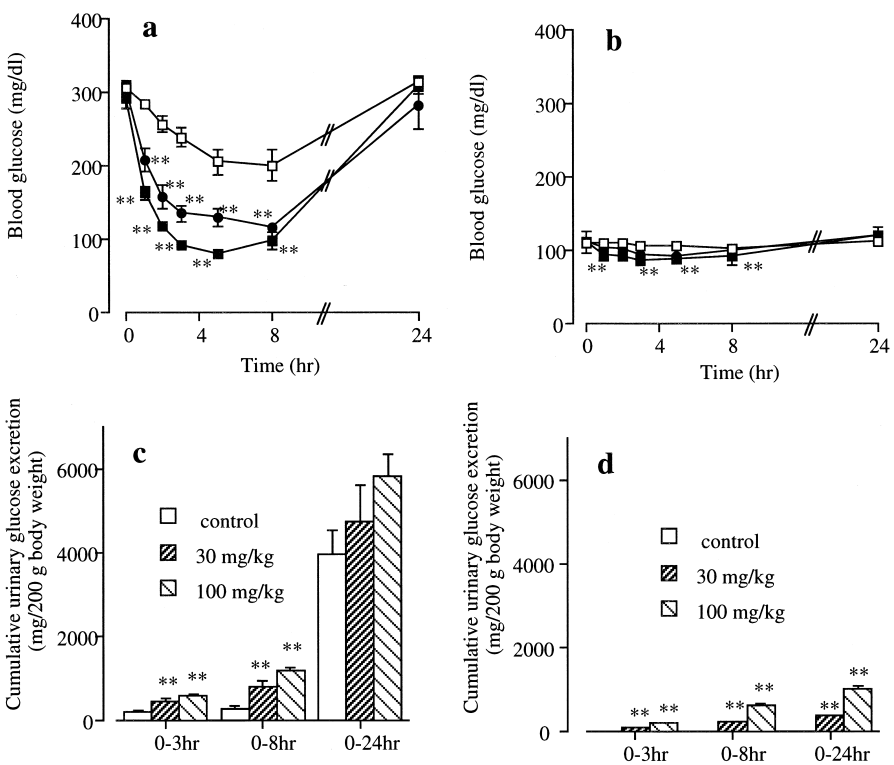

Fig. 4. Effect of single oral administration of T-1095 on blood glucose (a,b) and urinary glucose excretion (c,d) in diabetic (a,c) and normal (b,d) rats. T-1095 was administered to rats using an intragastric catheter, and changes in blood glucose and urinary glucose excretion were monitored for 24 h. □ Control; ● 30 mg/kg; ■ 100 mg/kg. Values are means ± SEM ($n = 8$). $^*P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control (by Dunnett's test).

2.10. Analytical methods

Blood glucose was determined with commercially available kits based on the glucose oxidase method (New Blood Sugar Test, Boehringer Mannheim, Germany). Plasma glucose levels in the clamp study and urinary glucose content were measured with a Glucose Analyzer (APEC, Danvers, MA, USA). HbA1c was determined with an affinity column method (Glyc-Affin-GHb®, Seikagaku, Tokyo, Japan). Plasma insulin levels were assayed using an enzyme-linked immunosorbent assay kit (Seikagaku) with rat insulin as the standard except in the clamp study. A radioimmunoassay kit (Insulin kit 'EIKEN', Eiken, Tokyo, Japan), with human insulin as the standard, was used for assay of plasma insulin in the clamp study. Plasma-free fatty acid, triglyceride, and acetoacetic acid contents were determined with enzymatic assay kits.

2.11. Data analyses

Data are expressed as means ± SEM. IC_{50} was calculated by non-linear least squares analysis using a four-parameter logistic model. In single- and continuous-administration studies, data were analyzed by two-way analysis of variance. Post hoc multiple comparisons were performed by Dunnett's method to compare each T-1095-treated group with the corresponding control (non-drug-treated) group. In the statistical analysis of skeletal muscle GLUT1 and GLUT4 contents, the data for normal and T-1095-treated diabetic groups were compared with those for the untreated diabetic group by Dunnett's method. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of T-1095 and T-1095A on Na^+-glucose cotransporter activity in kidney brush border membrane vesicles of normal and diabetic rats

Since preliminary studies indicated that T-1095A, but not T-1095, is mainly detectable in plasma following T-1095 administration (p.o.) to rats (Oku et al., 1999), we determined the effects of these compounds on Na^+-glucose cotransporter activity in vitro. Both T-1095 and T-1095A inhibited Na^+-dependent glucose uptake in brush border membrane vesicles prepared from renal tissues of normal (Fig. 3a) and diabetic rats (Fig. 3b). The IC_{50} values for Na^+-glucose cotransporter activity with T-1095, T-1095A, and phlorizin were 5.3, 0.5, and 1.0 μmol/l in normal rats, and 4.5, 0.4, and 0.8 μmol/l in diabetic rats, respectively. In terms of IC_{50} values, T-1095A was approximately 10 times more potent than T-1095. There was no significant difference in the inhibitory activities of these compounds between normal and diabetic rats.

3.2. Effect of single oral dose of T-1095 on blood glucose and urinary glucose excretion

Oral administration of T-1095 to diabetic rats resulted in a dose-dependent lowering of blood glucose levels (Fig. 4a). The antihyperglycemic effect was significant at a dose of 30 mg/kg. The maximal dose of 100 mg/kg induced a sustained decrease in blood glucose levels, which persisted for 8 h, but no significant reduction was detectable after 24 h. In contrast, there was only a marginal effect on blood glucose levels in normal rats even at 100 mg/kg of T-1095 (Fig. 4b).

Concurrently, there was a dose-dependent increase in urinary glucose excretion upon T-1095 administration in both diabetic and normal rats (Fig. 4c and d). The increase was more pronounced in diabetic rats than in normal rats; for example, 225.0 mg/200 g body weight of excess urinary glucose was excreted in 3 h with 30 mg/kg of T-1095 in diabetic rats; whereas only 94.3 mg/200 g body weight was excreted with the same T-1095 dose in normal rats.

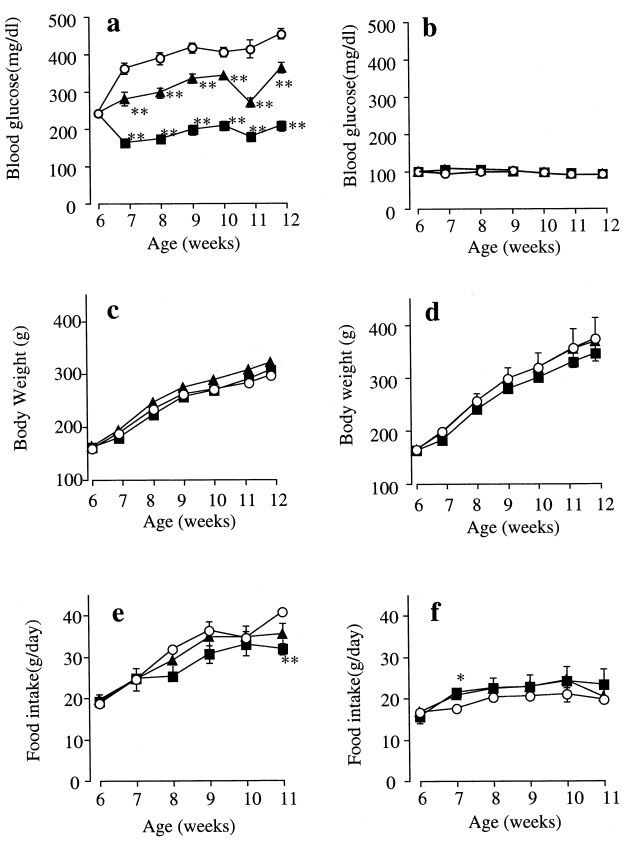

Fig. 5. Effect of continuous oral administration of T-1095 on blood glucose level (a,b), body weight (c,d), and food intake (e,f) in diabetic (a,c,e) and normal (b,d,f) rats. T-1095 was administered as a dietary admixture. ○ Control; ▲ 0.03%; ■ 0.1%. Each value represents the means ± SEM ($n = 8$). $^*P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control (by Dunnett's test).

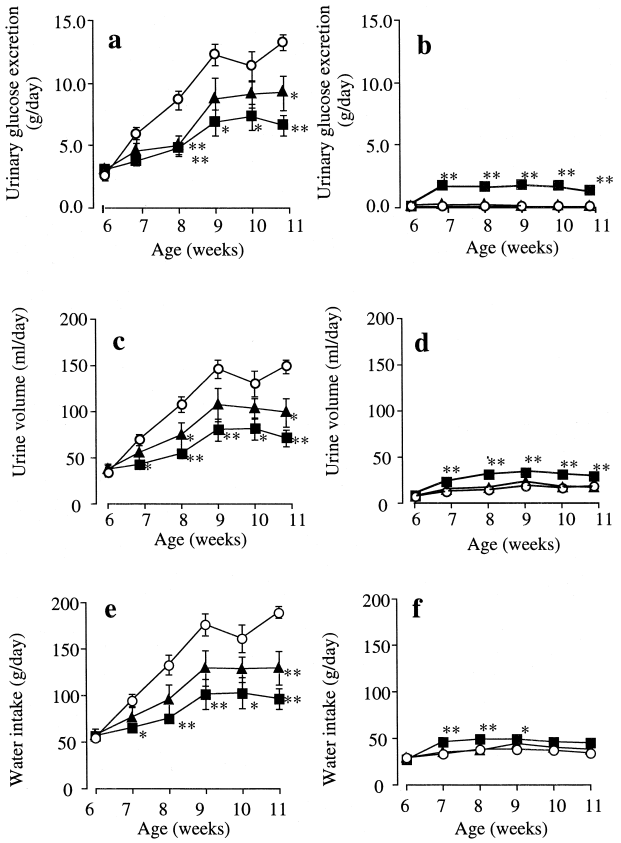

Fig. 6. Effect of continuous administration of T-1095 on urinary glucose excretion (a,b), urine volume (c,d), and water intake (e,f) in diabetic (a,c,e) and normal (b,d,f) rats. T-1095 was administered as a dietary admixture. ○ Control; ▲ 0.03%; ■ 0.1%. Values are means ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control (by Dunnett's test).

3.3. Effect of continuous administration of T-1095

3.3.1. Effect on physiological parameters

Next, we investigated the effect of continuous T-1095 treatment in both normal and diabetic rats. The calculated doses of the drug treatment groups were as follows: T-1095, 0.03%, 24.2 ± 0.7 (normal), 36.3 ± 3.2 (diabetic), T-1095, 0.1%, 88.5 ± 8.5 (normal), 120.0 ± 4.9 (diabetic) (mg · $kg^{-1} \cdot day^{-1}$, means ± SEM, $n = 8$). Diabetic animals ingested about 150% of the dose of normal animals, due to their hyperphagia.

Throughout the experimental period, both low (0.03%) and high (0.1%) doses of T-1095 lowered blood glucose levels in diabetic rats (Fig. 5a). In contrast, T-1095 did not affect the blood glucose levels in normal rats (Fig. 5b). Although the body-weight gain in diabetic rats was smaller than that in normal rats (Fig. 5c and d), there was apparent hyperphagia (Fig. 5e and f). T-1095 did not affect body weight in either normal or diabetic animals, but slightly suppressed the hyperphagia at 11 weeks of age (Fig. 5e) in diabetic rats. In contrast, the high dose of T-1095 slightly increased food intake at 7 weeks of age (Fig. 5f).

Glucosuria, polyuria, and polydipsia were marked in diabetic rats as compared with normal rats (Fig. 6). T-1095 increased urinary glucose excretion (Fig. 6b), urine volume (Fig. 6d), and, at some time points, water intake in normal rats (Fig. 6f). In contrast, urinary glucose excretion (Fig. 6a), urine volume (Fig. 6c), and water intake (Fig. 6e) in diabetic rats were dose-dependently suppressed by T-1095.

Table 1 summarizes the effects of T-1095 treatment on HbA1c, plasma insulin, plasma triglyceride, free fatty acid, and acetoacetic acid levels in diabetic and normal rats. Plasma triglyceride levels did not differ significantly between normal and diabetic rats. However, HbA1c, free fatty acid, and acetoacetic acid levels in diabetic rats were markedly higher than those in normal rats. T-1095 treatment reduced these parameters in diabetic, but not in normal rats. In addition, the plasma insulin level was lower in diabetic than in normal rats. T-1095 treatment improved hypoinsulinemia in diabetic rats, but did not affect plasma insulin levels in normal rats.

3.3.2. Effect on glucose tolerance and insulin secretion

Blood glucose levels, plasma insulin levels, and the area under the curve (AUC) for these parameters in the oral glucose tolerance test are shown in Fig. 7. In diabetic rats, glucose tolerance and insulin secretion were severely impaired (Fig. 7a–d), and the AUC for the blood glucose level was increased, while that for plasma insulin was decreased as compared to normal rats (Fig. 7e and f).

Table 1
Effects of T-1095 on HbA1c, plasma insulin, triglyceride, free fatty acid, and acetoacetic acid levels in normal and diabetic rats
Values are means ± SEM ($n = 8$).

	T-1095 (%)	HbA1c (%)	Insulin (ng/ml)	Triglyceride (mg/dl)	Free fatty acid (mEq/l)	Acetoacetic acid (mmol/l)
Normal	–	4.54 ± 0.09	1.93 ± 0.08	159.2 ± 11.7	302.6 ± 23.5	101.0 ± 4.8
	0.03	4.53 ± 0.04	2.02 ± 0.11	148.5 ± 15.2	292.6 ± 28.1	102.9 ± 3.0
	0.1	4.68 ± 0.08	1.78 ± 0.16	159.4 ± 23.6	228.8 ± 14.1	105.3 ± 2.6
Diabetic	–	12.64 ± 0.20	0.47 ± 0.07	161.9 ± 22.7	421.4 ± 54.2	338.1 ± 86.4
	0.03	10.56 ± 0.31[a]	0.99 ± 0.15[a]	153.3 ± 11.2	320.7 ± 27.9	203.9 ± 14.5
	0.1	7.23 ± 0.20[a]	1.18 ± 0.14[a]	120.3 ± 5.6	255.2 ± 15.6[a]	154.1 ± 10.4[b]

[a] $P < 0.01$ vs. the corresponding control (by Dunnett's test).
[b] $P < 0.05$ vs. the corresponding control (by Dunnett's test).

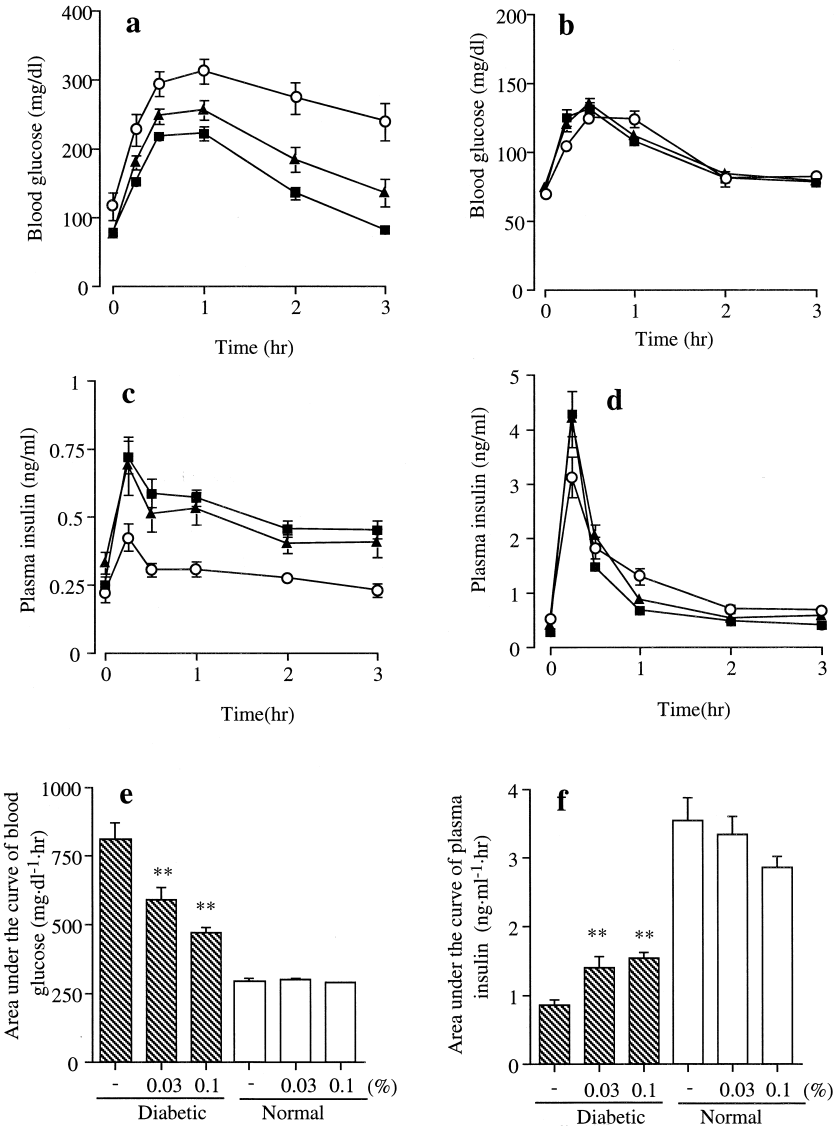

Fig. 7. Effect of continuous T-1095 treatment on blood glucose (a,b), plasma insulin (c,d), and AUC for blood glucose (e) and plasma insulin (f) during oral glucose tolerance test in diabetic (a,c) and normal (b,d) rats. ○ Control; ▲ 0.03%; ■ 0.1%. Each value represents the means ± SEM ($n = 8$). $^{**}P < 0.01$ vs. the corresponding control (by Dunnett's test).

Continuous T-1095 treatment significantly ameliorated the glucose intolerance and the insulin secretion deficiency in diabetic rats, while affecting neither parameter in normal rats.

Table 2
Plasma insulin, plasma glucose, glucose infusion rate, glucose utilization rate, glucose production rate, and urinary glucose loss in steady state during hyperinsulinemic euglycemic clamp studies in T-1095-treated and untreated rats, both diabetic and normals
Values are means ± SEM ($n = 5$ for normal groups, or 6 for diabetic groups).

	T-1095	Plasma insulin (mU/ml)	Plasma glucose (mg/dl)	Glucose infusion rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	Glucose utilization rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	Glucose production rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	Urinary glucose loss ($mg \cdot kg^{-1} \cdot min^{-1}$)
Normal	–	2.9 ± 0.1	108.9 ± 0.8	17.5 ± 1.0	17.6 ± 1.1	0.06 ± 0.15	0.01 ± 0.00
	0.03	2.8 ± 0.2	106.4 ± 2.0	17.2 ± 1.1	17.2 ± 1.1	0.01 ± 0.29	0.01 ± 0.00
	0.1	3.0 ± 0.5	108.5 ± 0.7	18.6 ± 1.0	18.7 ± 1.1	0.04 ± 0.16	0.01 ± 0.00
Diabetic	–	2.8 ± 0.2	107.3 ± 1.5	9.9 ± 0.5	12.7 ± 0.6	2.86 ± 0.11	0.01 ± 0.01
	0.03	2.9 ± 0.3	107.1 ± 1.9	13.4 ± 0.9[a]	14.1 ± 0.8	0.73 ± 0.52[b]	0.04 ± 0.03
	0.1	2.8 ± 0.2	107.9 ± 0.8	16.5 ± 1.2[b]	16.8 ± 0.9[b]	0.26 ± 0.33[b]	0.02 ± 0.01

[a] $P < 0.05$ vs. the corresponding control (by Dunnett's test).
[b] $P < 0.01$ vs. the corresponding control (by Dunnett's test).

3.3.3. Effect on insulin sensitivity in the glucose clamp study

The in vivo insulin action was quantitatively determined with the hyperinsulinemic euglycemic clamp technique. During the clamp study, steady-state plasma glucose, plasma insulin, and urinary glucose loss did not differ significantly among the groups (Table 2). In comparison with normal rats, there were decreases in the steady-state glucose infusion rate and GUR and an increase in GPR in diabetic rats (Table 2). T-1095 treatment normalized glucose infusion rate, GUR and GPR in diabetic rats, but did not influence these parameters in normal rats (Table 2). R'_g in skeletal muscle (quadriceps femoris), liver, and brown adipose tissue were decreased in diabetic rats. High doses of T-1095 significantly elevated the R'_g, i.e., restored the normal level, in skeletal muscle (Fig. 8b). T-1095 produced no significant changes in the R'_g in other tissues (Fig. 8a, c and d).

3.3.4. GLUT4 content of skeletal muscles

Fig. 9 shows a representative Western blot of immunoreactive GLUT1 and GLUT4 proteins in the crude membrane derived from skeletal muscle of normal, diabetic, and T-1095-treated (0.1%) diabetic rats. Identical amounts of protein (20 μg/well) were applied to the gels. Total GLUT1 content did not differ significantly among the groups. However, the total GLUT4 content in muscles of diabetic rats was decreased to 72.2% ($P < 0.01$) of that in normal rats, and T-1095 treatment normalized the GLUT4 content of diabetic rat muscle as reflected by an increase of 38.3% ($P < 0.01$).

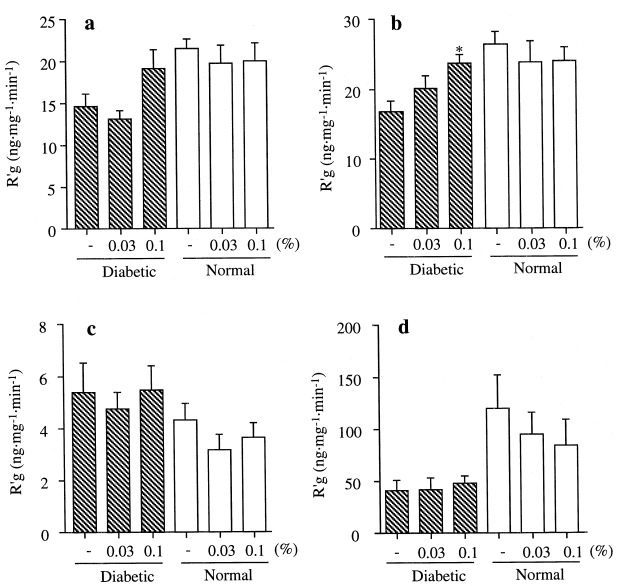

Fig. 8. Effect of continuous T-1095 administration on glucose utilization index in liver (a), skeletal muscle (quadriceps) (b), white adipose tissue (c), and brown adipose tissue (d), measured during hyperinsulinemic euglycemic clamp studies in normal and diabetic rats. Values are means ± SEM ($n = 5$ for normal groups, or 6 for diabetic groups). $^{*}P < 0.05$ vs. the corresponding control (by Dunnett's test).

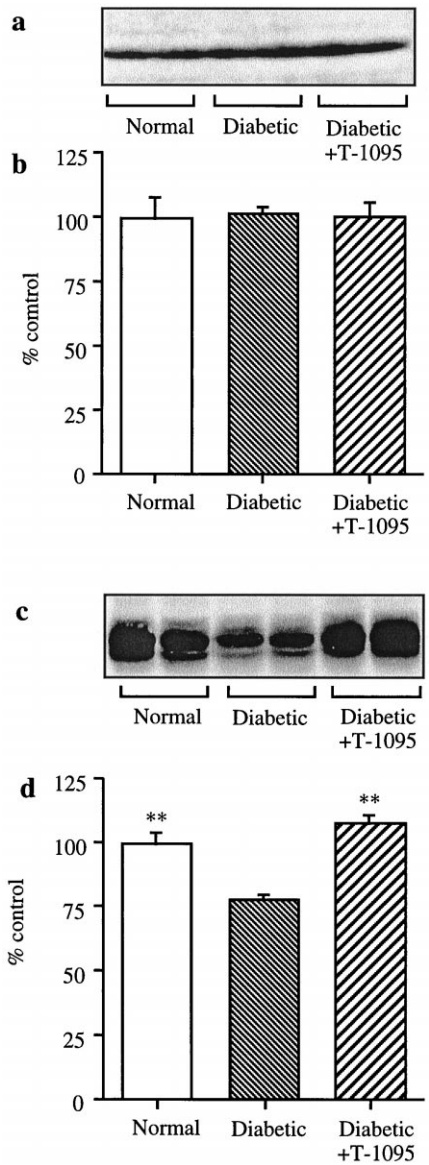

Fig. 9. Effects of T-1095 on contents of GLUT1 and GLUT4 proteins in skeletal muscles of diabetic rats. Crude membrane was prepared from skeletal muscles of normal, diabetic, and T-1095-treated diabetic rats as described in Section 2. Twenty micrograms of protein was applied to gels and GLUT1 (a,b), and GLUT4 (c,d) proteins were determined individually by immunoblotting as described in Section 2. Data are means ± SEM ($n = 4$). $^{**}P < 0.01$ vs. untreated diabetic rat (by Dunnett's test).

4. Discussion

Plasma glucose is filtered in glomeruli and then reabsorbed in epithelial cells of the renal proximal tubules via Na^+-glucose cotransporters (Silverman and Turner, 1992; Deetjen et al., 1995). Phlorizin, a specific inhibitor of

Na^+-glucose cotransporter, is reported to promote the excretion of glucose into the urine and lower blood glucose levels in several diabetic animal models upon subcutaneous injection (Rossetti et al., 1987; Blondel et al., 1990; Lisato et al., 1992; Crofford, 1995; Khan and Efendic, 1995; Krook et al., 1997). However, to date, there have apparently been no attempts to use phlorizin or any of its derivatives as antidiabetic agents. We speculate that several concerns, regarding the promise of these agents for therapeutic usage, have been raised. First, low oral bioavailability hampers the use of phlorizin as an oral antihyperglycemic agent. Second, diabetes is defined as a disorder presenting with hyperglycemia caused by both deficient insulin secretion and insulin resistance. Thus, hyperglycemia has been regarded primarily as a consequence, and diabetes treatment had two main targets: increase systemic insulin and normalize insulin resistance. Third, there is the fixed idea that increased urinary excretion of glucose is problematic and diabetes therapy should therefore decrease urinary glucose excretion. Furthermore, there are other concerns that an agent increasing urinary glucose excretion would normalize the blood glucose concentration; however, the increased urinary glucose secretion may induce polyuria possibly leading to dehydration, and ultimately various adverse effects including malnutrition.

T-1095, used in this study, is a phlorizin derivative, but unlike phlorizin, is absorbed effectively from the gut. After being absorbed into the bloodstream, T-1095 is changed to T-1095A via hepatic metabolism (Oku et al., 1999). Both T-1095A and T-1095 are inhibitors of Na^+-glucose cotransporter, though the potency of the former agent is approximately 10 times that of the latter and is nearly that of phlorizin, as shown in Fig. 3. Therefore, we consider that T-1095A primarily accounts for the antihyperglycemic effect of T-1095 in diabetic rats via renal Na^+-glucose cotransporter inhibition.

In normal rats, there was only a marginal glucose lowering effect of T-1095 with slight glucosuria on both single and chronic administration. Since Na^+-glucose cotransporters are abundantly expressed in proximal tubules, the transport capacity is approximately 3.5-fold that of glucose reabsorption at normal blood glucose levels (Deetjen et al., 1995). When glomerular glucose filtration increases in hyperglycemia, the reabsorption mechanism is saturated (i.e., all of Na^+-glucose cotransporters function at full capacity) and excess glucose overflows into the urine. Accordingly, inhibition of Na^+-glucose cotransporter is expected to increase urinary excretion of glucose and thus to reduce blood glucose levels more effectively under hyperglycemic than under normoglycemic conditions. Therefore, it is reasonable that T-1095 lowered the blood glucose levels more efficiently in a hyperglycemic than normoglycemic rats. We thus consider T-1095 to be a useful antihyperglycemic agent with a low risk of hypoglycemia.

It is well known that regardless of its underlying causes, hyperglycemia itself can impair both β cell function and peripheral glucose metabolism (glucose toxicity; Rossetti et al., 1990; Leahy et al., 1992). In fact, it has been reported that suppression of hyperglycemia by phlorizin ameliorates insulin resistance and β cell dysfunction in diabetic animal models (Rossetti et al., 1990). In addition, it is now clear that correction of hyperglycemia by any means results in apparent improvements in insulin secretion and resistance. In the present study, continuous treatment with T-1095 improved hyperglycemia and reduced HbA1c levels in diabetic rats. In addition, T-1095 produced a slight, but statistically significant improvement in hypoinsulinemia and glucose-induced insulin secretion on oral glucose tolerance testing, indicating partial recovery of β cell function. We speculate that β cell dysfunction in this model is due mostly to the toxic effect of streptozotocin and only partially to the additional effect of chronic hyperglycemia. Thus, the amelioration of glucose toxicity by T-1095 contributes to normalization only of the defect caused by the latter effect.

The improved insulin sensitivity in diabetic rats treated with T-1095 was confirmed, not only by the oral glucose tolerance test, but also by the data showing whole-body insulin action and insulin-stimulated suppression of hepatic glucose production. In addition, improvement of the impaired glucose utilization in skeletal muscles of diabetic rats was demonstrated in the hyperinsulinemic glucose clamp study. Since the rate-limiting step in glucose utilization by skeletal muscles under most metabolic conditions is glucose transport, the expression levels of GLUT1 and GLUT4 glucose transporters were investigated. In this study, the skeletal muscle GLUT4 content was lower in diabetic than in normal rats, and T-1095 normalized the GLUT4 content. Since a comparable effect of phlorizin has been reported in streptozotocin-treated rats (Dimitrakoudis et al., 1992a), the effect of T-1095 on GLUT4 content is likely to be mediated by the correction of hyperglycemia. In the light of a previous report showing that mice overexpressing GLUT4 are resistant to diabetes (Gibbs et al., 1995; Ikemoto et al., 1995), the normalization of the GLUT4 content is likely to, at least in part, explain the improved insulin sensitivity in muscle. As, presumably, a consequence of the recovery of both insulin secretion and insulin sensitivity, plasma free fatty acids, and ketone body (acetoacetic acid) levels in diabetic rats were significantly reduced by T-1095 treatment.

In contrast, T-1095 did not affect these physiological parameters or blood glucose levels in normal rats. Although T-1095 lowers the threshold of urinary glucose excretion in both normal and diabetic rats, the blood glucose levels in the former were low, such that only a very small amount of glucose was excreted into the urine with both single and continuous administration. The evidence that T-1095 has no effect on glucose metabolism in the non-diabetic rats suggests strongly that T-1095 amelio-

rated abnormal glucose metabolism by reducing hyperglycemia, without exerting direct effects on pancreatic β cells or insulin-sensitive tissues.

The important findings in this study are that continuous treatment with T-1095 decreased urinary glucose secretion and polyuria in diabetic rats, despite a single administration of T-1095 increasing urinary glucose excretion. These apparently conflicting findings are interesting and important with respect to the potential of T-1095 as an antidiabetic drug. We interpret this as follows: continuous administration of T-1095 improved insulin action by diminishing gluco-toxic effects on pancreatic β cells and peripheral tissues, as mentioned above. Thus, the blood glucose level was clearly normalized not only by the glucose excretory effect of T-1095, but also via increased insulin action. We speculate that the latter effect is predominant, such that there was less glucose excretion in the T-1095-treated group. Therefore, the possibility discussed above, that T-1095 may lead to polyuria and thereby dehydration, is unlikely. In addition, in spite of the improvement of hyperglycemia, there was no effect on weight gain, suggesting that a normal glucose level is adequate to maintain glucose metabolism even in the insulin-deficient diabetic rats.

5. Conclusion

We have shown that T-1095 is a potent antihyperglycemic agent, and that it improves whole-body glucose metabolism, insulin sensitivity, and β cell function. To date, no significant adverse effects have been observed in the diabetic animal model with the T-1095 at doses and periods used in this study. Thus, we suggest that oral administration of the Na^+-glucose cotransporter inhibitor, T-1095, is a potentially novel therapeutic approach to the management of diabetes mellitus. Clinical trials are needed to determine whether T-1095 has similar benefits in human diabetic subjects.

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