Hyperglycemia contributes insulin resistance in hepatic and adipose tissue but not skeletal muscle of ZDF rats

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IN NON-INSULIN-DEPENDENT diabetes mellitus (NIDDM), insulin resistance is an initiating pathogenic mechanism, and when pancreatic β-cells fail to secrete enough insulin to overcome insulin resistance, hyperglycemia becomes overt (38, 39, 40). However, hyperglycemia is not only a consequence of, but also an important factor in worsening both insulin resistance and insulin deficiency (4, 16, 38, 39, 40). Thus, in NIDDM patients whose glycemic control is poor, not only initiating factors such as obesity, high-fat diet, and insufficient exercise (23, 49), but also glucotoxicity, are regarded as factors contributing to the development of insulin resistance (12, 17). This study was designed to assess the contribution of hyperglycemia to insulin resistance in the liver, adipose tissue, and skeletal muscle in the Zucker diabetic fatty (ZDF) rat model.

ZDF rats are obese diabetic model animals that are polyphagic and hyperinsulinemic starting in the prehyperglycemic stage and then becoming hypoinsulinemic after establishment of hyperglycemia (42–44). This shift in disease phases is very similar to that seen in human NIDDM (2, 36). In this study, to remove the effect of hyperglycemia, a novel oral phlorizin derivative, T-1095, was administered to ZDF rats. An almost complete normalization of hyperglycemia was obtained at a high dosage of T-1095 without apparent changes in other factors, including overeating and obesity. By use of these T-1095-treated model animals, we investigated the extent to which hyperglycemia contributes to insulin resistance in the liver, adipose tissue, and skeletal muscle of ZDF rats. This is the first study to show clearly that the magnitude of the contribution of hyperglycemia to the development of insulin resistance differs among tissue types.

METHODS

Chemicals and analytical methods. T-1095 was synthesized at the Discovery Research Laboratory (Tanabe Seiyaku, Saitama, J apan). Plasma insulin levels were assayed by means of an ELISA kit (Seikagaku, Tokyo, J apan) with rat insulin as a standard. An RIA kit (insulin kit "Eiken," Eiken Chemical, Tokyo, J apan) with human insulin serving as the standard was used for the assay in the hyperinsulinemic euglycemic clamp study. Blood glucose levels were determined by means of commercially available kits based on the glucose oxidase method (New Blood Sugar Test, Boehringer Mannheim, Germany). Plasma glucose levels in the euglycemic clamp study and urinary glucose content were measured by a glucose analyzer (Apec, Danvers, MA). The hemoglobin A1c (HbA1c) level was determined by an affinity column method (Glyc-Affin, Seikagaku). All other chemicals were standard high-purity materials obtained from commercial sources.

Animals. ZDF (fa/f) and lean control (fa/+ ) rats were purchased from Charles River J apan at 6 wk of age, housed in stainless wire cages, and given normal laboratory chow (CE-2, Clea J apan, Tokyo, J apan) and water ad libitum. The

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experiments were started at 9 wk of age. The animals were divided into experimental groups matched for both body wt and blood glucose level. All experiments that used animals were approved by the Animal Care and Use Committee at Tanabe Selyaku.

For the single oral administration experiment, T-1095 was suspended in 0.1% hydrogenated castor oil polyethyleneglycol ether solution (Nikkol HCO-60, Nikko Chemical, Japan) and administered orally via stomach tube at a volume of 5 ml/kg. Blood samples were taken from the tail vein before, and 1, 2, 3, 5, 8, and 24 h after, drug administration to determine glucose levels. Urine samples were collected to measure glucose content by means of metabolic cages.

To achieve continuous administration, T-1095 was given as food admixtures. Both lean control and ZDF rats (9 wk old) were kept on a CE-2 diet containing 0.03 or 0.1% (wt/wt) food admixtures. Both lean control and ZDF rats (9 wk old) were divided into experimental groups matched for both body wt and glucose intolerance by use of the hyperinsulinemic euglycemic clamp study. A hyperinsulinemic euglycemic clamp study was performed after 4 wk of continuous T-1095 administration. The animals were fasted for 16 h before this experiment to assure that the drug had been entirely eliminated, and insulin sensitivity was then investigated by use of the hyperinsulinemic euglycemic clamp technique (20). In brief, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and catheters were inserted into both the right and left femoral veins for insulin and glucose infusion, respectively. A catheter in the left jugular vein was used for blood sampling and injection of [14C]glucose and deoxy-[2-3H]glucose. Regular human insulin (1.8 U·kg⁻¹·h⁻¹, Humulin R, Eli Lilly, Indianapolis, IN) was infused intravenously, and whole blood glucose concentrations were determined at 5-min intervals. A 10% glucose solution was infused to maintain the blood glucose concentration at 120 mg/dl. When the blood glucose level reached a steady-state level, [U-¹⁴C]glucose (Amersham Life Science, Buckinghamshire, UK) was administered as an initial intravenous priming dose (4 µCi), and immediately after by continuous infusion at a rate of 0.2 µCi/min. After a stabilization period of ≥30 min, the bladder was emptied and urine sampling was started (i.e., time = 0 min). The steady-state glucose infusion rate (SSGIR) was determined as the mean of values obtained during a 0- to 60-min period. At 15 min, 2-deoxy-[¹³C]glucose (50 µCi, Amersham Life Science) was infused intravenously as a bolus injection. Additional blood samples were collected for the determination of plasma tracer concentrations and insulin levels at 17, 20, 40, and 60 min, respectively.

At completion of the glucose clamp study (i.e., 60 min), urine in the bladder was collected to evaluate the rate of urinary glucose loss (UGL) during the 60-min study. The liver, skeletal muscle (quadriceps femoris), white adipose tissue (epididymal fat), and brown adipose tissue (interscapular fat) were rapidly removed and frozen in liquid nitrogen. Twenty to 200 mg of tissues were weighed and dissolved in Soluen-350 (Packard Jpn, Tokyo, Japan) and the 1H activity was measured. The rate of glucose disappearance (Rd) was calculated by dividing the [14C]glucose infusion rate (dpm·min⁻¹·kg body wt⁻¹) by the steady-state value of glucose-specific activity (dpm/mg). The whole body glucose utilization rate (GUR) and the hepatic glucose production rate (HGP) were calculated as follows: GUR = Rd – UGL, and HGP = Rd – SSGIR. The glucose utilization index (Rd), an estimate of tissue glucose uptake, was calculated as described by James et al. (20).

Glucokinase activity assay. Hepatic glucokinase (GK) activity was measured by the spectrophotometric continuous assay method (10). Liver homogenates were prepared in ice-cold buffer (pH 7.5) containing 50 mM HEPES, 250 mM sucrose, 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, and 2.5 mM dithioerythritol and centrifuged at 105,000 g for 60 min to sediment the microsomal fraction which was used for measuring glucose-6-phosphatase (G-6-Pase) activity. The enzyme activity of the supernatant (soluble fraction) was assayed in a buffer (pH 7.4) containing 50 mM HEPES, 7.5 mM MgCl₂, 100 mM KCl, 5 mM ATP, 2.5 mM dithioerythritol, 10 mg/ml BSA, 0.5 mM NAD⁺, 4 U/ml glucose-6-phosphate dehydrogenase (L. mesenteroides) and 0.5 mM for hexokinase or 50, 25, 12.5, and 6.25 mM glucose for total phosphorylating activity. The hexokinase reaction was initiated by adding ATP, and the rate of NAD⁺ reduction was recorded at 340 nm. Total phosphorylating activity was determined as the difference of absorbance change in the presence and absence of ATP. GK activity was calculated as the difference between the total phosphorylating activity and hexokinase activities.

G-6-Pase activity assay. The microsomal fraction was suspended in the homogenization buffer and diluted with ice-cold buffer (pH 6.5) containing 100 mM HEPES and 0.1 mM EDTA. The G-6-Pase reaction was initiated by adding 10, 5, 2.5, 1.25, and 0.625 mM glucose 6-phosphate at 30°C and stopped after 10 min with a 2.2-fold volume of the stop solution containing 3.7 mM ammonium molybdate and 240 mM SDS in 270 mM H₂SO₄. The absorption at 750 nm was monitored after color development by adding a 1/5 volume of 1.2 M ascorbic acid. Pₐ was used as a standard (26).

Preparation of isolated adipocytes from ZDF rats. ZDF and T-1095-treated ZDF rats were killed by decapitation after 5 wk of treatment. Epidymal fat pads were excised and digested with collagenase (1 mg/ml, Sigma, St. Louis, MO), and the isolated adipocytes were suspended in Krebs-Ringer bicarbonate (KRB) buffer supplemented with 30 mM HEPES, 1% BSA fraction V, 3 mM glucose, and 200 mM adenosine at pH 7.4 as described (9, 21). Adipocytes were prepared with a 25% adipocrit.

Subcellular fractionation and Western blotting. Isolated adipocytes were suspended in KRB buffer containing BSA (10 mg/ml, pH 7.4) and preincubated with or without insulin for 30 min at 37°C. After incubation, subcellular membrane fractions of adipocytes were prepared by differential centrifugation (9, 21). The homogenates were centrifuged at 3,000 g for 15 min to sediment the crude membrane fraction. The fat cakes were discarded, and the supernatant was centrifuged at 12,000 g for 15 min to sediment the plasma membrane (PM) fraction. The supernatant was centrifuged at 28,000 g for 15 min, and the resulting supernatant was centrifuged at 146,000 g for 75 min, yielding a particulate fraction termed low-density microsomes (LDM). Proteins (0.05 mg) in PM and LDM fractions were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T, vol/vol), and then incubated for 2 h at room temperature with antiserum to the COOH terminus of GLUT-4. After being washed with TBS-T, the bound immunoglobulins were probed with anti-rabbit IgG antibody. The membranes were immediately reacted with an enhanced chemiluminescence (ECL) reagent (Amersham Life Science), and the chemiluminescence level was quantified with a molecular imager GS-525 by use of Screen-HP.

Data analyses. The data are presented as means ± SE. Statistical analyses were performed by closed testing procedures. In brief, the untreated diabetic control group was...

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initially compared with the lean group by unpaired Student's t-test. When the difference between these two groups was significant, a multiple comparison was performed with Dunnett's test to compare each T-1095-treated group with the diabetic control group. P values \(<0.05\) were considered to be statistically significant.

RESULTS

Effect of a single administration of T-1095 on blood glucose and urinary glucose excretion. When administered orally, T-1095 is absorbed from the gut into the bloodstream and metabolized to an active form termed T-1095A. T-1095A suppresses the activity of SGLTs in the kidney, thereby inhibiting reabsorption of glucose (34, 46). Thus a dose-dependent increase in urinary glucose excretion was observed with the administration of T-1095 to ZDF rats (at 3 and 8 h after administration, as shown in Fig. 1A). Concurrently, T-1095 dose dependently lowered blood glucose levels in ZDF rats (Fig. 1B). The antihyperglycemic effect was apparent at a dose of 30 mg/kg, and the maximal dose of 100 mg/kg induced a sustained decrease in blood glucose levels for 24 h. In contrast, there was only a marginal effect on blood glucose levels in lean rats, even at a dosage of 100 mg/kg (Fig. 1C).

Effect of continuous treatment with T-1095 on hyperglycemia and physiological parameters. Continuous administration of T-1095 was shown to lower blood glucose levels significantly in ZDF rats at both low (0.03%) and high (0.1%) doses (Fig. 2A). In contrast, no such hypoglycemic effect of T-1095 was observed in lean rats (control lean rats, 86.3 ± 2.9 mg/dl, T-1095-treated lean rats, 86.1 ± 2.7 mg/dl). ZDF rats exhibited apparent hyperphagia and continued to weigh more than lean rats (Fig. 2B). T-1095 treatment did not affect the body wt increase of ZDF rats, despite slightly suppressing hyperphagia at 1 wk after the beginning of T-1095 administration (Fig. 2C).

Marked glycosuria, polyuria, and polydipsia in ZDF rats were shown to progress with aging (Fig. 3). Continuous treatment with T-1095 dose dependently suppressed the age-related increases in urine volume (Fig. 3A), urinary glucose excretion (Fig. 3B), and water intake (Fig. 3C) in ZDF rats.

Table 1 summarizes the effects of T-1095 treatment on various parameters including fasting plasma glu-
Each value indicates mean ± SE (n = 6). ** P < 0.01 vs. lean rat. * P < 0.05, † P < 0.01 vs. lean ZDF rat.

Table 1. Physiological parameters of nonfasting animals treated with or without T-1095 for 4 wk

<table>
<thead>
<tr>
<th>ZDF Rat Groups</th>
<th>T-1095</th>
<th>Animals, n</th>
<th>Fasting Values</th>
<th>Nonfasting Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>6</td>
<td>71.2 ± 1.6</td>
<td>0.41 ± 0.04</td>
<td>4.84 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>196.8 ± 25.7</td>
<td>3.18 ± 0.287</td>
<td>13.24 ± 0.233</td>
</tr>
<tr>
<td>T-1095 treated</td>
<td>0.03%</td>
<td>128.1 ± 9.4</td>
<td>4.52 ± 1.02</td>
<td>9.83 ± 0.53</td>
</tr>
<tr>
<td>T-1095 treated</td>
<td>0.1%</td>
<td>101.1 ± 3.4†</td>
<td>3.90 ± 0.26</td>
<td>5.25 ± 0.11</td>
</tr>
</tbody>
</table>

Values of plasma IRI indicate pretreatment and 4-wk treatment in all rats. IRI, immunoreactive insulin; ZDF, Zucker diabetic fatty rat. § P < 0.01, vs. lean ZDF rat, * P < 0.05, † P < 0.01, vs. control ZDF rat.
DISCUSSION

T-1095 is a derivative of phlorizin but, unlike phlorizin, it is absorbed efficiently from the gut into the bloodstream when administered orally. After entering the circulation, T-1095 is metabolized to an active form, termed T-1095A (33, 45), that suppresses the activity of renal SGLTs involved in glucose reabsorption in proximal tubules (22, 33, 34, 45). Thus orally administered, T-1095 exerts essentially the same effect as intravenously injected phlorizin, which cannot be absorbed from the gut (8). However, taken as a food admixture, T-1095 efficiently suppresses postprandial blood glucose elevation, which results in a more efficient antihyperglycemic effect than phlorizin when given as a continuous venous infusion (45).

The ZDF rat is regarded as an insulin-resistant diabetic model exhibiting obesity and overeating because of an abnormality of the leptin system (18, 32, 35). In our experiments, the nonfasting blood glucose levels of ZDF rats averaged 250 ± 10 mg/dl at 9 wk of age, and a defect in insulin secretion had already been indicated in an oral glucose tolerance test at 11 wk (data not shown). Although the molecular mechanisms underlying insulin resistance remain unclear, the major cause of insulin resistance in ZDF rats has been considered to be obesity related (3, 18, 32, 35, 41–44, 48). Although the blood glucose level was normalized, the fasting insulin level was not reduced by T-1095 treatment. The ZDF strain of rat is a substrain of the Zucker fatty strain that is obese, hyperinsulinemic, and normoglycemic. Thus we speculate that these Zucker-strain rats are genetically hyperinsulinemic irrespective of blood glucose levels, and that correction of glucose elevation, which results in a more efficient antihyperglycemic effect than phlorizin when given as a continuous venous infusion (45). The ZDF rat is regarded as an insulin-resistant diabetic model exhibiting obesity and overeating because of an abnormality of the leptin system (18, 32, 35). In our experiments, the nonfasting blood glucose levels of ZDF rats averaged 250 ± 10 mg/dl at 9 wk of age, and a defect in insulin secretion had already been indicated in an oral glucose tolerance test at 11 wk (data not shown). Although the molecular mechanisms underlying insulin resistance remain unclear, the major cause of insulin resistance in ZDF rats has been considered to be obesity related (3, 18, 32, 35, 41–44, 48). Although the blood glucose level was normalized, the fasting insulin level was not reduced by T-1095 treatment. The ZDF strain of rat is a substrain of the Zucker fatty strain that is obese, hyperinsulinemic, and normoglycemic. Thus we speculate that these Zucker-strain rats are genetically hyperinsulinemic irrespective of blood glucose levels, and that correction of glucose elevation, which results in a more efficient antihyperglycemic effect than phlorizin when given as a continuous venous infusion (45).

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hyperglycemia does not, therefore, reduce hyperinsulinemia in ZDF rats.

In the present study, despite the degree of obesity being unchanged, the normalization of hyperglycemia with T-1095 treatment ameliorated insulin resistance significantly, as shown by a reduction in GIR in the hyperinsulinemic euglycemic clamp study. In fact, T-1095 treatment slightly improved total GUR. On the other hand, the elevated triglyceride (TG), free fatty acid (FFA), and total cholesterol (TCHO) values in ZDF rats were not altered by T-1095 treatment. We speculate that these levels are not related to abnormal leptin metabolism, because there is a functional leptin receptor defect in ZDF rats. In fact, plasma leptin levels in T-1095-treated ZDF rats were similar to those in untreated ZDF rats (data not shown). Thus it is very likely that the improved insulin sensitivity achieved with T-1095 treatment is attributable to removal of the effect of hyperglycemia (so-called glucotoxicity).

Of great interest in this study is the observation that the degree of insulin sensitivity improvement differs among tissues after blood glucose normalization. Insulin resistance in the liver was markedly suppressed by normalizing blood glucose levels. In contrast, although insulin resistance in adipose tissues was also improved significantly, this normalization was partial. Finally, insulin resistance in muscle was apparently not improved. These results show clearly that the contribution of hyperglycemia to insulin resistance differs in these three insulin-sensitive tissues.

First, T-1095 treatment profoundly reversed both reduced glucose uptake and elevated glucose production in the liver, as demonstrated by the hyperinsulinemic euglycemic clamp study. Although abrupt correction of hyperglycemia might have counterregulated HGP in ZDF rats during the glucose clamp study, elevations in fasting glucose level and the ratio of GK to G-6-Pase activities agree well with the result of the glucose clamp study. GK and G-6-Pase play important roles in blood glucose regulation. GK and G-6-Pase,

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**Fig. 6.** Effects of continuous oral administration of T-1095 on hepatic glucokinase (GK) (A), glucose-6-phosphatase (G-6-Pase) (B), and GK/G-6-Pase (C) activities in nonfasting ZDF and lean rats. Each value indicates mean ± SE (n = 6). *P < 0.05, **P < 0.01 vs. lean rat. *P < 0.05, **P < 0.01 vs. ZDF control rat.

![Image](https://via.placeholder.com/150)

**Fig. 7.** Effects of continuous oral administration of T-1095 on GLUT-4 protein contents in plasma membrane (A, B) and low-density microsomes (C, D) obtained from isolated adipocytes of ZDF and lean rats. Isolated adipocytes were stimulated with 10⁻⁶ M insulin for 15 min at 37°C. Each value indicates mean ± SE (n = 4).
both of which are known to be regulated by insulin, convert glucose to glucose 6-phosphate, and glucose 6-phosphate to glucose through glucose production, respectively (19, 30, 46, 47). In our experiment, the reduced GK/G-6-Pase activity ratio in ZDF rats was corrected by continuous T-1095 treatment. Hepatic GK activity and G expression levels are reportedly increased in streptozotocin-treated (STZ) rats, and phlorizin treatment ameliorated the impaired GK activity (6, 27); therefore, it is likely that hyperglycemia altered GK and G-6-Pase activities, and that T-1095 treatment corrected the enzyme activities through amelioration of hyperglycemia.

Second, in adipose tissues, the normalization of insulin resistance with T-1095 treatment was demonstrated by the increased R values in the glucose clamp study. It is well known that the rate-limiting step for glucose uptake into adipocytes is governed by the amount of GLUT-4 on the cell surface (11, 24, 28, 37). In fact, the translocation of GLUT-4 protein from LDM to PM was markedly impaired in adipose tissues of ZDF rats, and this impairment was shown to be ameliorated by T-1095 treatment. However, the degree of normalization was partial, such that factors worsening insulin sensitivity were still present. We speculate that the enlargement of adipocytes is likely to be one such factor, because it has been reported that larger adipocytes possess lower insulin sensitivity in glucose transport activity (29).

Finally, the reduced skeletal muscle R of ZDF rats was not restored significantly by T-1095 treatment. A previous study demonstrated that impaired insulin sensitivity in STZ diabetic rats was improved by long-term correction of hyperglycemia with phlorizin (5). Thus, although in most nongenetic diabetic models (e.g., STZ rats) hyperglycemia is likely to be a major cause of the development of insulin resistance in peripheral tissues (5–7, 27, 30, 31, 38), glucotoxicity is not involved in the mechanism underlying insulin resistance in ZDF rat muscles (1, 51). Moreover, it was also reported that insulin resistance was not improved in muscle of ZDF rats treated with pioglitazone, an insulin sensitizer of the peroxisome proliferator-activated receptor- (PPAR- ) agonist (13). On the contrary, it has been reported that the PPAR -agonist improves muscle insulin resistance (51) and -glucosidase inhibitor recovers muscle GLUT-4 contents in ZDF rats (15). In the present study, T-1095 reduced hyperglycemia without affecting plasma TG, FFA, and total cholesterol. These results suggest that insulin resistance in skeletal muscle is already developed by other mechanisms including lipotoxicity when hyperglycemia is established, and that hyperglycemia does not further increase insulin resistance, in ZDF rats. Hawkins et al. (16) have suggested that the increased availability of glucose and FFA affects insulin-induced glucose uptake via a common mechanism. In the present study, FFA levels were elevated in ZDF rats, whereas muscle TG contents were not determined. Therefore, although we speculate that the impaired insulin sensitivity in ZDF rat muscle may be related to an abnormality in lipid metabolism as well as the leptin system, further study is necessary to clarify this issue.

In summary, insulin resistance in hepatic and adipose tissues is induced by glucotoxicity resulting from long-term hyperglycemia in ZDF rats. In contrast, insulin resistance in skeletal muscles is not due to glucotoxicity. Taken together, these raise the possibility of the tissue dependency of glucotoxicity not only in other animal models but also in individual diabetic patients (14). However, although hyperglycemia is a consequence of insufficient insulin secretion and insulin resistance, these results suggest that normalization of hyperglycemia leads to an apparent improvement in insulin resistance. Thus hyperglycemia should be considered not only a consequence of but also a major factor in worsening insulin sensitivity. We now consider T-1095 to be a potentially novel therapeutic strategy for diabetes management, because it evidently reduces hyperglycemia-induced insulin resistance in liver and adipose tissues. Further study is required to elucidate the molecular mechanisms underlying insulin resistance in the skeletal muscle of ZDF rats.

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