Original Article

Glucose Toxicity Is Responsible for the Development of Impaired Regulation of Endogenous Glucose Production and Hepatic Glucokinase in Zucker Diabetic Fatty Rats

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The effect of restoration of normoglycemia by a novel sodium-dependent glucose transporter inhibitor (T-1095) on impaired hepatic glucose uptake was examined in 14-week-old Zucker diabetic fatty (ZDF) rats. The nontreated group exhibited persistent endogenous glucose production (EGP) despite marked hyperglycemia. Gluconeogenesis and glucose cycling (GC) were responsible for 46 and 51% of glucose-6-phosphatase (G6Pase) flux, respectively. Net incorporation of plasma glucose into hepatic glycogen was negligible. Glucokinase (GK) and its inhibitory protein, GK regulatory protein (GKRP), were colocalized in the cytoplasm of hepatocytes. At day 7 of drug administration, EGP was slightly reduced, but G6Pase flux and GC were markedly lower compared with the nontreated group. In this case, GK and GKRP were colocalized in the nuclei of hepatocytes. When plasma glucose and insulin levels were raised during a clamp, EGP was completely suppressed and GC, glycogen synthesis from plasma glucose, and the fractional contribution of plasma glucose to uridine diphosphoglucose flux were markedly increased. GK, but not GKRP, was translocated from the nucleus to the cytoplasm. Glucotoxicity may result in the blunted response of hepatic glucose flux to elevated plasma glucose and/or insulin associated with impaired regulation of GK by GKRP in ZDF rats. *Diabetes* 55:2479–2490, 2006

Acute elevation in plasma glucose levels and the subsequent rise in plasma insulin levels exert a direct inhibitory effect on glucose production and a stimulatory effect on glucose uptake, both of which contribute to the maintenance of euglycemia in nondiabetic individuals (1). Patients with type 2 diabetes exhibit preprandial hyperglycemia and excessive postprandial hyperglycemia. The preprandial hyperglycemia is due to impaired suppression of endogenous glucose production (EGP) in response to elevated plasma glucose and/or insulin levels as well as impaired glucose uptake in peripheral tissues (2–4). In addition to these impairments, a defect of splanchnic glucose uptake in response to elevated plasma glucose and insulin contributes to excessive postprandial hyperglycemia (5,6). The mechanisms by which the reduction of insulin production and loss of glucose effectiveness in the regulation of hepatic glucose flux are developed in type 2 diabetes remain to be clarified.

Chronic hyperglycemia (also referred to as glucose toxicity) per se has been proposed as an independent factor in the development of insulin resistance in skeletal muscle and adipose tissue and in the reduction of the ability of pancreatic β-cells to respond to an acute glycemic challenge in type 2 diabetes (7–9). The contribution of chronic hyperglycemia to the deteriorating changes in hepatic glucose metabolism has been implied in some diabetic models. Nawano et al. (10) administered Zucker diabetic fatty (ZDF) rats a novel orally bioavailable inhibitor of the sodium-dependent glucose transporter (T-1095) from 9 weeks of age, the time when fasting hyperglycemia first became evident. The animals did not experience marked hyperglycemia and exhibited alleviated development of impairment of insulin's ability to suppress EGP. Impaired insulin-induced suppression of EGP observed in muscle GLUT4 knockout mice also was normalized when hyperglycemia was reduced by treating with phlorizin from 12 to 20 weeks of age (11). The maintenance of normoglycemia for 72 h by treatment with insulin restored the normal effectiveness of glucose to suppress EGP in poorly controlled diabetic subjects (12). These results suggested a role for glucose toxicity in the development of impaired insulin-mediated suppression of net hepatic glucose production (NHGP) in diabetes. On the other hand, hyperinsulinemia did not stimulate substantial net hepatic glucose uptake (NHGU) in the presence of euglycemia (13–16). NHGU and subsequent storage of glucose as glycogen were substantially increased in the presence of hyperglycemia combined with hyperinsulinemia (14–16). Net hepatic glucose flux is the balance between the rate of glucose phosphorylation catalyzed by glucokinase (GK) and the rate of dephosphorylation of glucose-6-phosphate (G-6-P) catalyzed by glucose-6-phosphatase (G6Pase). It has been suggested that a defect in HGU in response to the elevated plasma glucose and insulin levels observed in type 2 diabetes results, at least partly, from the failure of an increase in the plasma glucose concentration to enhance the flux through GK (4,17).

Functional GK activity in the liver is regulated by

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EGP, endogenous glucose production; FFA, free fatty acid; G-6-P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GC, glucose cycling; GK, glucokinase; GKRP, GK regulatory protein; GNG, gluconeogenesis; GST, glutathione-S-transferase; HGP, hepatic glucose production; HGU, hepatic glucose uptake; NHGP, net HGP; NHGU, net HGU; PEP, phosphoenolpyruvate; UDP, uridine diphosphophate; UDP-G, uridine diphosphoglucose.

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changes in the amount of enzyme present due to alteration in its gene transcription, allosteric inhibition by GK regulatory protein (GKRP), and translocation between the nucleus and cytoplasm (18,19). To clarify whether glucose toxicity plays a role in the development of impaired HGU and GK activity in diabetes, we examined the effect of the reduction of hyperglycemia by treatment with T-1095 for 7 days on hepatic glucose flux and expression and intracellular localization of GK and GKRP and GK translocation in hepatocytes in 14-week-old ZDF rats, a model of obese type 2 diabetes.

RESEARCH DESIGN AND METHODS

Six-week-old male ZDF (ZDF/GmiCrl-*fa/fa*) rats were purchased from Charles River Laboratories (Wilmington, MA) and were fed Formulab Diet 5008 (Purina LabDiet; Purina Mills, Richmond, IN) in an environmentally controlled room with a 12-h light/dark cycle. Surgery was performed to place sterile silicone rubber catheters in the ileal vein, the left common carotid artery, and the right external jugular vein at 11 weeks of age as previously described (20,21).

At 13 weeks of age, the animals, which had >21 mmol/l blood glucose level after a 6-h fast, were divided randomly into three experimental groups: the control group fed Formulab Diet 5008 ad libitum, the T-1095 group fed Formulab Diet 5008 containing 0.1% (wt/wt) T-1095 (Tanabe Seiyaku, Saitama, Japan) ad libitum for 7 days, and the paired-fed group given daily the amount of the diet that matched the food intake in the T-1095 group but without T-1095. Blood samples were taken through the jugular vein catheter at 9 A.M. on days 0 (before starting to feed), 2, 4, and 7. On day 7, the animals were fasted for 6 h and used for either a refeeding or glucose kinetic study. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of both the U.S. Department of Agriculture and National Institutes of Health. All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

In the refeeding experiment, the animals in the T-1095 treatment and paired-fed groups were allowed access to the Formulab Diet 5008 with and without compound 0.1% (wt/wt) T-1095, respectively, for 60 min. The animals were allowed to access water freely. Blood samples were taken through the arterial catheter.

In the glucose kinetic studies, each study consisted of a 2-h tracer equilibration (−180 to −60 min) and a 1-h control (−60 to 0 min), followed by a 2-h test period (0–120 min). At −180 min, both [2-^{3}H] and [3-^{3}H] glucose were given at 60 μCi in a bolus through the jugular vein catheter followed by continuous infusion at 0.6 μCi/min. In the basal studies, during the test period the animals were kept without additional treatment. In the clamp studies, somatostatin was infused through the jugular vein catheter at 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ in order to inhibit endogenous insulin and glucagon secretion. Insulin and glucagon were infused into the hepatic portal circulating system through the ileal vein catheter at 6 $mU \cdot kg^{-1} \cdot min^{-1}$ and 2.6 $ng \cdot kg^{-1} \cdot min^{-1}$, respectively. Plasma glucose levels were maintained at ~24 mmol/l by infusion of 50% glucose solution through the jugular vein catheter at a variable rate. At 110 min, [U-^{14}C] alanine was given at 200 μCi in a bolus through the arterial catheter followed by continuous infusion at 10 μCi/min. Blood samples were obtained via the arterial catheter. Blood glucose levels were monitored using HemoCue (HemoCue, Mission Viejo, CA). To maintain the hematocrit levels, erythrocytes were washed with saline, resuspended in saline, and readministered to each animal. At the end of each experiment, the animal was anesthetized with an intravenous infusion of sodium pentobarbital (40 mg/kg) and a laparotomy was performed immediately. The median lobe of the liver was excised and dropped into ice-cold PBS for immunohistochemical analysis. The left lobe of the liver and skeletal muscle (vastus lateralis) were frozen in situ using Wollenberger tongs precooled in liquid nitrogen. This procedure took <20 s from the point of successful anesthesia.

Enzyme activities. For GK, hexokinase, and G6Pase activities measurements, 200 mg of freeze-clamped livers were homogenized in 2 ml of buffer containing 50 mmol/l HEPES, 100 mmol/l KCl, 1 mmol/l EDTA, 5 mmol/l $MgCl_2$, and 2.5 mmol/l dithioerythritol (22). Homogenates were centrifuged at 100,000*g* for 45 min to sediment the microsomal fraction. The postmicrosomal fraction (10 μl) was assayed in a medium (1 ml), pH 7.4 at 37°C, containing 50 mmol/l HEPES; 100 mmol/l KCl; 7.5 mmol/l $MgCl_2$; 5 mmol/l ATP; 2.5 mmol/l dithioerythritol; 10 mg/ml albumin; 0.5, 8, and 100 mmol/l glucose; 0.5 mmol/l NAD^+; and 4 units/ml of G-6-P dehydrogenase (*Leuconostoc mesenteroides*). The reaction was initiated by the addition of ATP, and the rate of NAD^+ reduction was recorded at 340 nm for 30 min. Glucose phosphorylation by hexokinase was determined as the absorbance change in the presence of 0.5 mmol/l glucose minus the absorbance change in the absence of glucose under conditions in which the absorbance was increasing linearly between 10 and 30 min. Glucose phosphorylation by GK was determined as the absorbance change in the presence of 8 or 100 mmol/l glucose minus the absorbance change in the presence of 0.5 mmol/l glucose under conditions in which the absorbance was increasing linearly between 10 and 30 min. G6Pase activity was measured as described by Lange et al. (23). The sedimental fraction (50 μl) was incubated with 0.5, 1.0, 2.5, 5.0, and 10.0 mmol/l G-6-P. The reaction was carried out at 37°C and stopped after 20 min with a solution containing acid molybdate, with two-ninths volumes of 10% SDS and one-ninth volume of 10% ascorbic acid. It was then incubated for 1 h at 37°C, and the absorbance was read at 820 nm. A standard curve was constructed using different concentrations of Pi. Glycogen synthase and phosphorylase activities in the liver were measured using the method described by Golden et al. (24).

Immunostaining and Western blot analysis for GK and GKRP. The immunostaining for GK and GKRP expression and the quantitative image analysis of the immunofluorescences were performed as previously reported (20,21). Briefly, one of serial sections (4 mm) was incubated with an equivolume mixture of sheep anti-rat glutathione-S-transferase (GST)-GK serum and rabbit anti-rat GST-GKRP serum, and the other was incubated with an equivolume mixture of preimmune sheep and rabbit serum. After being washed with 0.1% Triton X-100/PBS, these serial sections were incubated with Cy3-conjugated donkey anti-sheep IgG and Cy5-conjugated donkey anti-rabbit IgG as well as YoPro-1. Quantitative image analysis of Cy3 (GK) and Cy5 (GKRP) immunofluorescence in the nucleus and the cytoplasm of hepatocytes was performed using a Zeiss LSM 510 confocal laser scanning microscope. The internal He/Ne laser and external argon-krypton laser were used at 543, 647, and 488 nm that optimally excite Cy3, Cy5, and YoPro-1 fluorescence, respectively. After the transfer of image files to a Power Macintosh Imaging workstation, the image was converted to tag image file format, and the individual cells were quantified using nuclear/cytoplasmic pixel density rationing with National Institutes of Health Image (v. 1.56). Three and five microscopic areas were randomly selected in the section incubated with the preimmune and immune serum, respectively. In the first step, to detect clear Cy3 and Cy5 immunofluorescence signals in the nucleus, we selected cells that exhibited the immunoreactive area with YoPro-1 (a cross-sectional area of the nucleus) above 18 × 18 pixel area. In the second step, to assess nonspecific (background) immunofluorescence, Cy3 and Cy5 immunofluorescence in the nucleus and cytoplasm were measured in all the selected cells in the selected microscopic area of the section incubated with preimmune serum. In the third step, to quantify GK distribution and translocation, we selected cells with a higher immunofluorescence intensity of Cy5 (GKRP) in the nucleus compared with the serial section stained with preimmune serum. A total of 10–20 cells were Cy5 positive in each microscopic field, and thus the total number of the chosen cells was 50–100 cells for each section. For each cell, a round, 18 × 18 pixel area (181 square pixels) was analyzed in the nucleus and the cytoplasm by measuring mean pixel density (range 0–255 gray-scale levels). The ratios of nuclear to cytoplasmic fluorescence of GK and GKRP were determined by digital image analysis, using Scion Image and averaged for each animal. The average value was normalized to that in the standard liver sample, stained on the same day. The results for each group were expressed as the means ± SE of the normalized value in five animals. Western blot analyses of GK and GKRP were performed with the anti-rat GST-GK and GST-GKRP sera that were used for immunohistological staining described above.

Metabolites in blood and tissue. The glycogen in liver and skeletal muscle, G-6-P concentration in liver, plasma triglycerides and nonesterified free fatty acids (FFAs), insulin, and glucagon; blood lactate; and alanine were determined as previously described (21).

To determine the specific activity of [2-^{3}H] and [3-^{3}H] glucose, plasma was deproteinized using $Ba(OH)_2$ and $ZnSO_4$. After centrifugation, the supernatant was passed through cation and anion (Dowex 50 W × 8 and Amberlite IRA-67, respectively; Sigma-Aldrich, St. Louis, MO) exchange columns to remove [^{14}C]-labeled intermediates. [2-^{3}H] and [3-^{3}H] glucose radioactivity in plasma glucose and glycogen glucose were determined by selective enzymatic detritiation of [2-^{3}H] glucose (25). External standards of [2-^{3}H] and [3-^{3}H] glucose suspended in control rat plasma were processed in parallel with each assay to calculate the degree of detritiation of each isotope during each sample plasma assay. Overall completion of detritiation of [2-^{3}H] glucose was 97.2 ± 0.4%, while 99.8 ± 0.3% of [3-^{3}H] glucose remained intact. Plasma specific activity of [2-^{3}H] and [3-^{3}H] glucose (dpm/μmol) were determined by the method of DeBodo et al. (26).

The liver contents of uridine 5′-diphosphate-glucose (UDP-G) and UDP-galactose were obtained through two sequential chromatographic separations according to a modification (21) of the method of Giaccari and Rossetti (27). The amount of [^{3}H] and [^{14}C] radioactivity in each fraction was measured.

To determine the concentration and [^{14}C] radioactivity of phosphoenolpyruvate (PEP) in the liver, frozen liver samples (750 mg) were homogenized

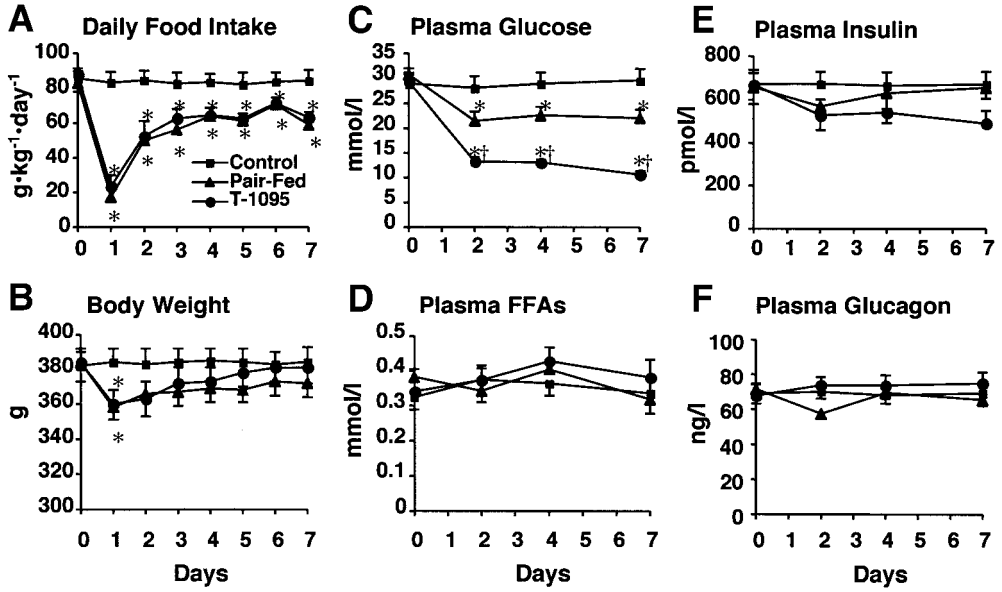

FIG. 1. Daily food intake (*A*), body weight (*B*), and arterial plasma levels of glucose (*C*), nonesterified FFAs (*D*), insulin (*E*), and glucagon (*F*) under nonfasting conditions before and during test feeding periods. ■, control group; ▲, paired-fed group; ●, T-1095 treatment group. Values are means ± SE for 10 rats in each group. **P* < 0.05 vs. the control group. †*P* < 0.05 vs. the paired-fed group.

in 1 ml of ice-cold 0.6 mol/l perchloric acid. Following centrifugation for 5 min at 3,000*g*, the supernatants were neutralized with 100 μl of 2 mol/l KOH and of 0.5 mol/l triethanolamine at 0°C, centrifuged for 2 min at 3,000*g*, and then the supernatants were diluted with 4 ml of 20 mmol/l NaCl. The diluted supernatant was allowed to drip through an anion exchange column (AGI-X8 200-400 mesh [4-ml volume]; Bio-Rad, Hercules, CA), which was previously washed with 30 ml of 1 N HCl followed by 30 ml H_2O and then 30 ml of 20 mmol/l NaOH. After washing of the column with 4 ml of 20 mmol/l NaCl, 4 ml of 100 mmol/l NaCl, 2 × 4 ml of 200 mmol/l NaCl, and then 4 ml of 100 mmol/l HCl, PEP was eluted with 7 ml of 200 mmol/l HCl. The fraction of 200 mmol/l HCl was dried using a vacuum dryer (Savant Instruments, Farmingdale, NY) and was reconstituted with 0.4 ml of high-performance liquid chromatography running buffer (100 mmol/l KH_2PO_4, 100 mmol/l K_2HPO_4, and 10 mmol/l tetrabutyl ammonium hydrogen phosphate), adjusted to pH 6.05 with orthophosphoric acid, filtered, and degassed with a 0.45-μm fier. This reconstituted fraction contained >93% of PEP compared with a standard spiked with [^{14}C] PEP. Chromatography was carried out using an high-performance liquid chromatography LC-18-T Supelcosil strong anion-exchange column (250 × 4.6 mm i.d.) in series from Supelco with 1.0 ml/min of flow rate at 35°C by programmable gradient. Absorbance was measured at 200 nm at 1.0 absorbance unit full scale.

Calculations. Rates of [2-^{3}H] and [3-^{3}H] glucose–determined glucose turnover were calculated as the ratio of the rate of infusion of [2-^{3}H] and [3-^{3}H] glucose (dpm/min) and the [2-^{3}H] and [3-^{3}H] specific activity in plasma glucose, respectively, according to the steady-state equations of Steele et al. (28): [2-^{3}H] glucose turnover rate = infusion rate of [2-^{3}H] glucose/plasma specific activity of [2-^{3}H] glucose and [3-^{3}H] glucose turnover rate = infusion rate of [3-^{3}H] glucose/plasma specific activity of [3-^{3}H] glucose, respectively. EGP was determined as the difference between [3-^{3}H] glucose turnover rate and the glucose infusion rate: EGP = [3-^{3}H] glucose turnover rate − glucose infusion rate. To estimate the amount of [2-^{3}H] and [3-^{3}H] glucose incorporated into hepatic and skeletal muscle glycogen via the direct pathway (GLY-[2-^{3}H] glucose and GLY-[3-^{3}H] glucose, respectively), [2-^{3}H] and [3-^{3}H] radioactivities incorporated into glycogen were divided by the [2-^{3}H] and [3-^{3}H] specific activity of plasma glucose, respectively: GLY-[2-^{3}H] glucose = [2-^{3}H] radioactivity in glycogen/plasma specific activity of [2-^{3}H] glucose and GLY-[3-^{3}H] glucose = [3-^{3}H] radioactivity in glycogen/plasma specific activity of [3-^{3}H] glucose, respectively. The fractional detritiation of [2-^{3}H] G-6-P ($D_{[2\text{-}3H]}$) by exchange reaction of [^{3}H] of [2-^{3}H] G-6-P with [H^+] of H_2O mediated by hexose isomerase was calculated as the ratio of GLY-[2-^{3}H] glucose to GLY-[3-^{3}H] glucose: $D_{[2\text{-}3H]}$ = 1 − GLY-[2-^{3}H] glucose/GLY-[3-^{3}H] glucose. This calculation is based on the assumption that the ratio of [2-^{3}H] to [3-^{3}H] glucose incorporated into glycogen approximates that of the G-6-P pool. Glucose cycling (GC) is defined as input of extracellular glucose into the G-6-P pool followed by exit of plasma-derived G-6-P back into the extracellular pool: GC = ([2-^{3}H] glucose turnover rate − [3-^{3}H] glucose turnover rate)/$D_{[2\text{-}3H]}$. The in vivo flux through G6Pase (G6Pase-flux) were calculated as the sum of EGP plus GC: G6Pase-flux = EGP + GC. The fractional contribution of plasma glucose via the direct pathway (UDPG-Glu) and PEP (UDPG-PEP) to UDP-G flux were calculated as the ratio of [3-^{3}H] specific activity and [^{14}C] specific activity in hepatic UDP-G to [3-^{3}H] specific activity in plasma glucose and [^{14}C] specific activity in hepatic PEP, respectively: UDPG-Glu = ([^{3}H] specific activity of hepatic UDP-G/[3-^{3}H] specific activity in plasma glucose) × ([3-^{3}H] radioactivity in hepatic glycogen/total [^{3}H] radioactivity in hepatic glycogen) and UDPG-PEP = [^{14}C] specific activity in UDP-glucose/(2 × [^{14}C] specific activity in hepatic PEP). Total glycogen synthesis (GLY) in the liver was measured as the product of GLY-[3-^{3}H] glucose and UDPG-Glu: GLY = UDPG-Glu/UDPG-Glu. The amount of PEP incorporated into hepatic glycogen (GLY-PEP) was measured as the product of GLY and UDPG-PEP: GLY × UDPG-PEP. Gluconeogenic flux from PEP was calculated as the G6Pase-flux × UDPG-PEP.

Statistical analyses. Data are expressed as means ± SE. For the time-course data, the significance of differences between groups was analyzed using two-way repeated-measures ANOVA. Otherwise, significant differences between groups were analyzed using one-way ANOVA or Student's *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

Effects of T-1095 and paired feeding on plasma glucose, insulin, glucagon, and FFA levels. In the control group, daily food intake (83 ± 2 g · kg^{-1} · day^{-1}) and plasma glucose, triglycerides, FFAs, lactate, alanine, insulin, and glucagon levels were stable during the test feeding period (Fig. 1). In the T-1095 group, the daily food intake and body weight decreased by 75 and 7%, respectively, on day 1, after which these values gradually recovered to 80 and 100% of their original levels, respectively, by day 4. Plasma glucose levels were markedly reduced from 29.3 ± 1.5 to 13.2 ± 1.0 mmol/l by day 2 and reached 10.6 mmol/l at day 7. The daily food intake in the paired-fed group was well matched to that in the T-1095 group. Plasma glucose levels remained at ~22 mmol/l during the test feeding period. Interestingly, plasma insulin, glucagon, FFAs, blood lactate, and alanine levels were not altered by the paired feeding and T-1095 treatment.

To assess whether T-1095 treatment was able to prevent postprandial hyperglycemia, the feeding test was performed in the paired-fed and T-1095 treatment groups after a 6-h fast on day 7 (Fig. 2). In the paired-fed group, the

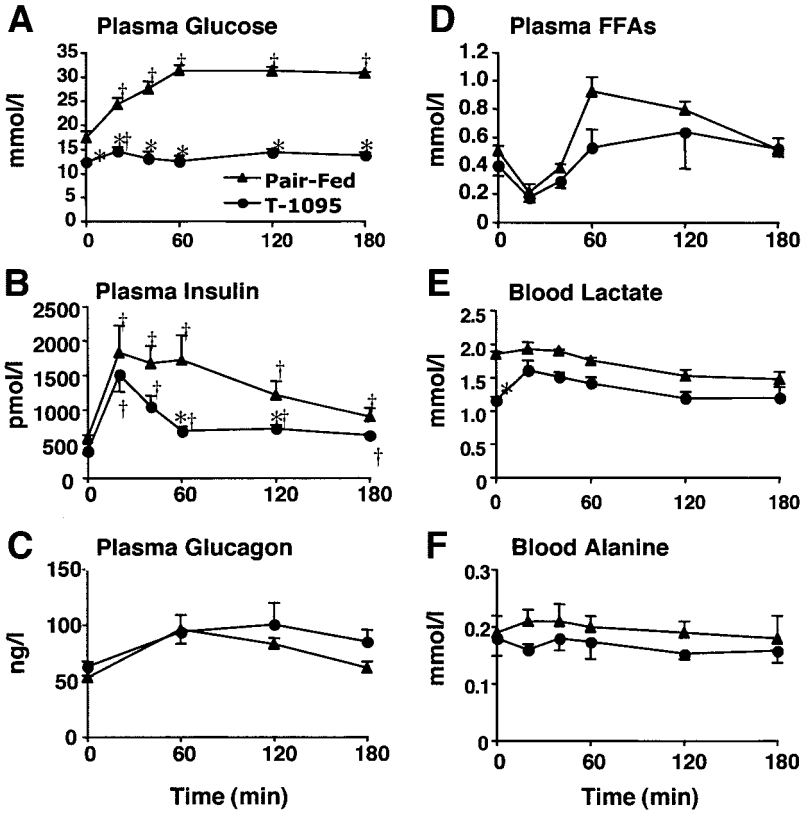

FIG. 2. Changes in plasma glucose (*A*), insulin (*B*), glucagon (*C*), FFAs (*D*), blood lactate (*E*), and alanine (*F*) with refeeding of normal chow in 6-h–fasted paired-fed rats (▲) and of chow containing 0.1% T1095 in T-1095–treated rats (●). Values are means ± SE for five rats in each group. *$P < 0.05$ vs. the paired-fed group. †$P < 0.05$ from the values at 0 min in the identical group.

animals consumed 20.9 ± 2.0 g/kg during the 1-h feeding period. Plasma glucose and insulin levels increased to 32 mmol/l and 1,700 pmol/l, respectively, at the end of the feeding period. In the T-1095 treatment group, while the food intake (31.5 ± 3.4 g/kg body wt) was even higher, plasma glucose levels were not significantly increased and plasma insulin levels were increased only transiently during the refeeding period. Changes in plasma glucagon, FFAs, blood lactate, and alanine levels were similar between the groups.

Glucose kinetics under basal conditions. The control group exhibited marked hyperglycemia (22.5 ± 1.6 mmol/l) and hyperinsulinemia (551 ± 47 pmol/l). The [2-^{3}H] and [3-^{3}H] glucose turnover rates and the difference between the turnover rates were stable at 78.1 ± 6.3, 47.6 ± 5, and 30.4 ± 4.9 μmol · kg^{-1} · min^{-1}, respectively, during the control and the test period (Fig. 3). As shown in Table 1, the fractional contribution of plasma glucose via the direct pathway and PEP to UDP-G flux were 50 and 46%, respectively, and the fractional contribution of the other sources, including glycogenolysis, G-6-P from glycerol, and glucose futile cycle through triose phosphate to UDP-G flux was <4%. GC was responsible for 50% of G6Pase flux, and gluconeogenesis (GNG) through PEP was responsible for 41% of G6Pase flux and 90% of EGP. In the paired-fed group, while plasma glucose levels (17.8 ± 2.2 mmol/l) were lower, measured parameters in glucose kinetics were not statistically different when compared with those in the control group. In the T-1095 treatment group, compared with the other groups, even in the presence of lower plasma glucose (10.8 ± 1.0 mmol/l) and insulin (405 ± 41 pmol/l), [3-^{3}H] glucose turnover rate (40.1 ± 3.8 μmol · kg^{-1} · min^{-1}) was slightly lower and [2-^{3}H] glucose turnover rate (59.2 ± 6.9 μmol · kg^{-1} · min^{-1}) and the difference between [2-^{3}H] and [3-^{3}H] glucose turnover rate (17.8 ± 4.1 μmol · kg^{-1} · min^{-1}) were markedly lower. There was a significant decrease in G6Pase flux that was due to markedly decreased GC. G-6-P content in the liver was not different among the three groups. Glucose excretion into urine during the study was similar among the control, paired-fed, and T-1095 treatment groups (10.7 ± 5.1, 11.6 ± 6.8, and 7.5 ± 4.9 μmol · kg^{-1} · min^{-1}, respectively).

Glucose kinetics during hyperglycemic-hyperinsulinemic clamp. When arterial plasma glucose and insulin levels were clamped at ~24 mmol/l and 850 pmol/l, the glucose kinetic parameters measured in the control group were not changed from that in the control period (Fig. 4, Table 1) and were not statistically different from those under basal conditions (Fig. 3 and Table 1). In the paired-fed group, while [2-^{3}H] and [3-^{3}H] glucose turnover rates were increased (Fig. 4), EGP, G6Pase flux, GC, and the fractional contribution of plasma glucose via the direct pathway and PEP were not significantly different from that during the test period in the basal study of the identical group and in the clamp study in the control group (Table 1). In the T-1095 treatment group, compared with the other groups, both [2-^{3}H] and [3-^{3}H] glucose turnover rate and the difference between the turnover rates were markedly higher, and EGP was completely suppressed. While G6Pase flux was not different, the fractional contribution of plasma glucose to UDP-G flux and GC was 25% and five times higher, respectively. Conversely, the fractional contribution of PEP to UDP-G flux and GNG through PEP was significantly lower. Glucose excretion into urine during

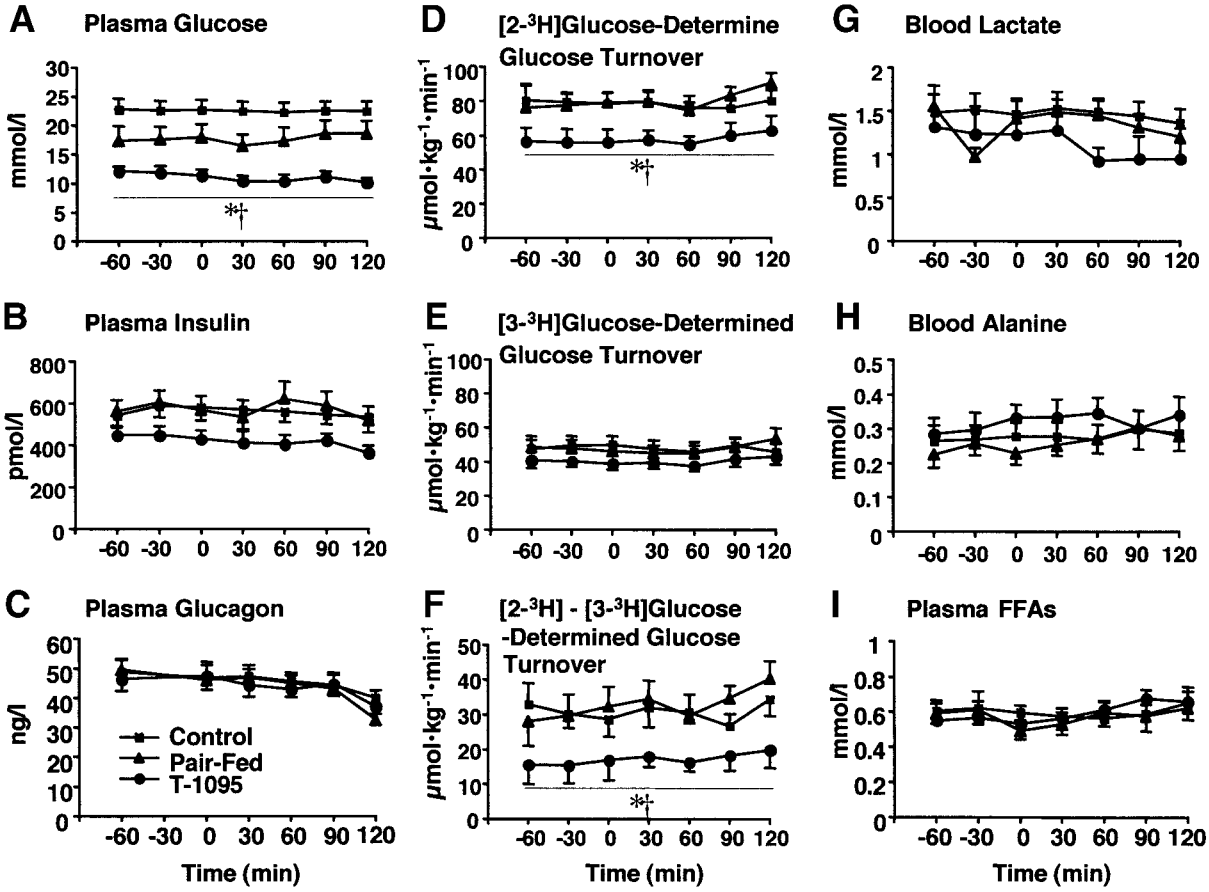

FIG. 3. Plasma glucose (*A*), insulin (*B*), glucagon (*C*), [2-^{3}H] glucose (*D*), [3-^{3}H] glucose (*E*), GC rate (*F*), blood lactate (*G*), alanine (*H*), and plasma FFAs (*I*) under basal conditions. ■, control group; ▲, paired-fed group; ●, T-1095 treatment group. Values are means ± SE for five rats in each group. **P* < 0.05 vs. the control group. †*P* < 0.05 vs. the paired-fed group.

the clamp study was higher in the T-1095 treatment group (21.1 ± 5.3 μmol · kg^{-1} · min^{-1}) than in the control and paired-fed groups (11.1 ± 5.3 and 9 ± 1.0 μmol · kg^{-1} · min^{-1}, respectively). Endogenous glucose disappearance rates (μmol · kg^{-1} · min^{-1}), which were calculated by subtracting the glucose excretion rate into urine from

TABLE 1
Hepatic glucose flux in the control, paired-fed, and T-1095 groups in basal conditions and under hyperglycemia-hyperinsulinemic clamp

	Basal			Clamp		
	Control	Paired fed	T-1095	Control	Paired fed	T-1095
Fractional contribution to flux through UDP-G						
Plasma glucose (%)	50.1 ± 5.5	51.3 ± 3.8	34.9 ± 5.1*†	47.8 ± 5.1	44.4 ± 4.0	58.2 ± 2.3*†‡
PEP (%)	46.2 ± 3.6	45.2 ± 3.9	51.0 ± 8.2	45.2 ± 5.2	41.5 ± 4.8	20.7 ± 3.1*†‡
Others (%)	3.8 ± 2.2	3.5 ± 2.8	14.1 ± 6.2	7.0 ± 4.2	14.1 ± 5.4	21.1 ± 4.6
[2-^{3}H]/[3-^{3}H] in glycogen	0.27 ± 0.05	0.28 ± 0.03	0.27 ± 0.03	0.25 ± 0.04	0.18 ± 0.08	0.52 ± 0.11*†‡
G6Pase flux (μmol · kg^{-1} · min^{-1})	89.3 ± 7.2	94.7 ± 6.6	64.7 ± 7.6*†	99.6 ± 8.9	94.2 ± 9.2	116.4 ± 11.3
EGP (μmol · kg^{-1} · min^{-1})	47.6 ± 5.0	47.6 ± 4.8	40.1 ± 3.8	50.0 ± 6.5	53.0 ± 8.0	−6.1 ± 13.0*†‡
GNG (μmol · kg^{-1} · min^{-1})	41.3 ± 4.5	42.8 ± 3.0	33.0 ± 3.9	45.0 ± 6.2	39.1 ± 5.5	23.9 ± 5.3*†
GC (maximum) (μmol · kg^{-1} · min^{-1})	41.6 ± 6.7	47.1 ± 6.0	24.6 ± 5.6*†	49.6 ± 11.6	41.2 ± 8.6	122.5 ± 12.3*†‡
G-6-P (nmol/g liver)	121 ± 13	116 ± 9	124 ± 13	125 ± 12	134 ± 13	137 ± 16
Hepatic glycogen content (μmol glucose/g liver)	300 ± 25	282 ± 24	220 ± 15*†	295 ± 35	321 ± 9	201 ± 28*†
Incorporation into glycogen (μmol glucose/g liver)						
From plasma glucose	5.7 ± 1.8	6.5 ± 2.1	5.5 ± 1.5	8.4 ± 2.2	14.0 ± 3.7‡	40.4 ± 4.5*†‡
From PEP	5.2 ± 1.6	5.7 ± 1.8	8.1 ± 2.2	6.5 ± 2.4	9.9 ± 3.6	13.8 ± 3.2

Data are means ± SE for five rats in each group. *Significant difference from the corresponding values in the control group under identical experimental condition ($P < 0.05$). †Significant difference from the corresponding values in the paired-fed group under identical experimental condition ($P < 0.05$). ‡Significant difference from the corresponding values in the identical group in basal condition ($P < 0.05$).

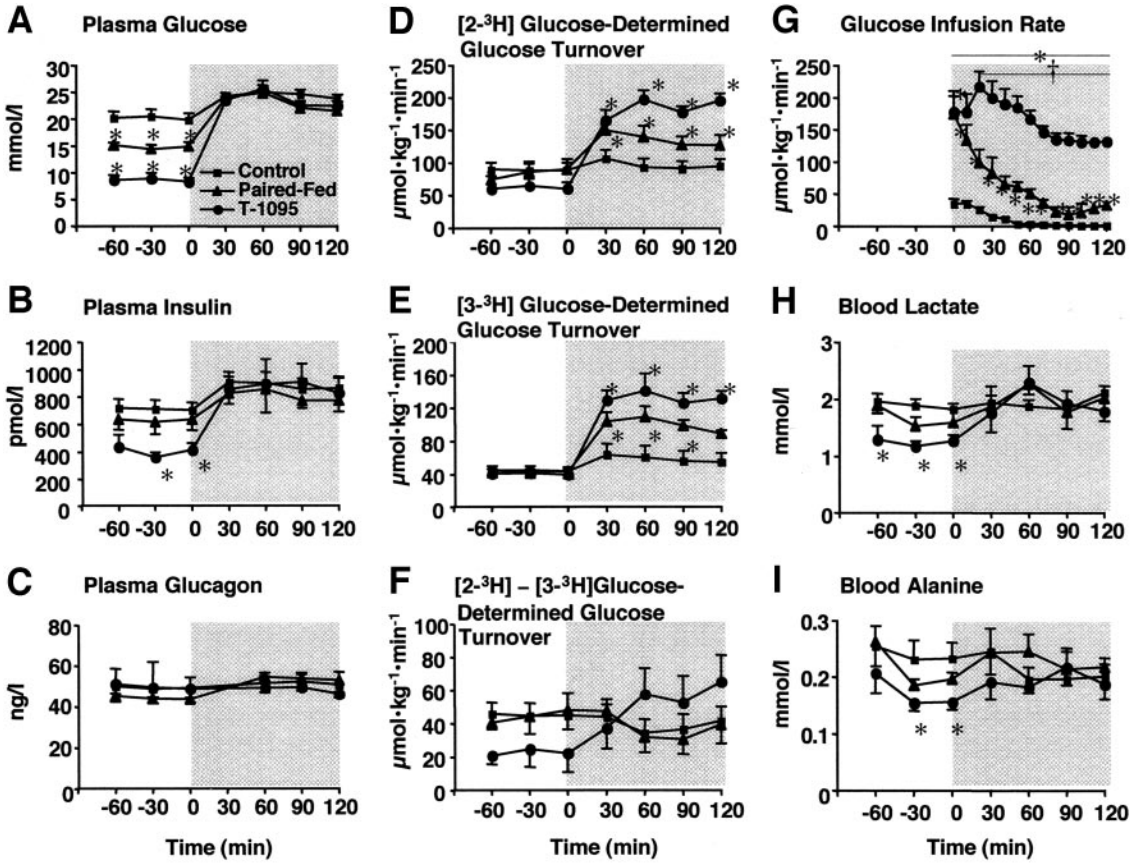

FIG. 4. Plasma glucose (*A*), insulin (*B*), alanine glucagon (*C*), glucose turnover rates determined with [2-^{3}H] glucose (*D*) and [3-^{3}H] glucose (*E*), GC rate (*F*), glucose infusion rate (*G*), blood lactate (*H*), and alanine (*I*) before and during hyperglycemic-hyperinsulinemic clamp. ■, control group; ▲, paired-fed group; ●, T-1095 treatment group. Values are means ± SE for five rats in each group. **P* < 0.05 vs. the control group. †*P* < 0.05 vs. the paired-fed group.

[3-^{3}H] glucose turnover rates (58.3 ± 13.2 in the control, 100.5 ± 9.3 in the paired-fed, and 131.4 ± 13.3 μmol · kg^{-1} · min^{-1} in the T-1095 group as an average during the clamp period), were still significantly higher in the paired-fed (91.5 ± 9.0) and in the T-1095 group (110.3 ± 11.8) than in the control group (47.2 ± 11.2). G-6-P content in the liver was not different among the three groups.

Glycogen content and glycogen synthesis in liver and skeletal muscle. Hepatic glycogen contents at the end of the basal and clamp studies were significantly lower in the

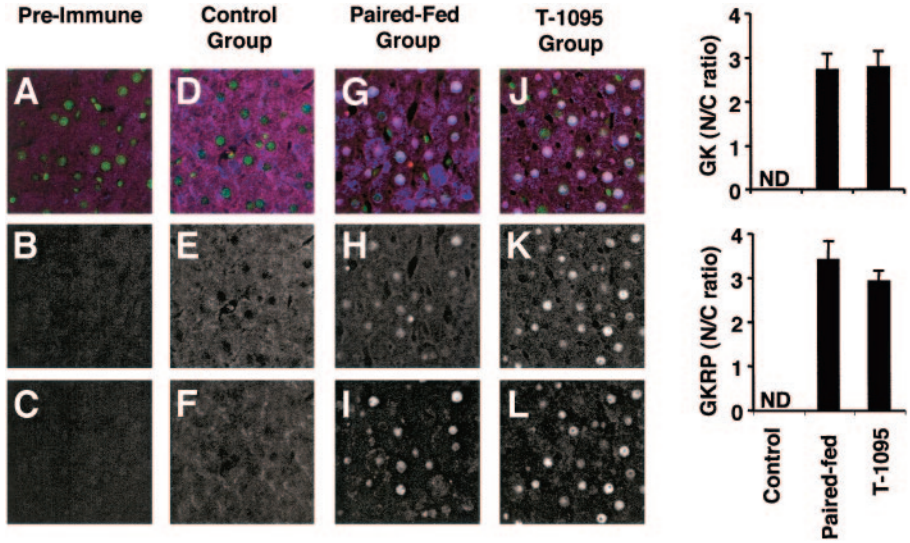

FIG. 5. The intracellular distribution of GK and GKRP in hepatocytes at the end of each basal studies. *A*, *D*, *G*, and *J*: Immunofluorescence stainings for GK and GKRP are revealed as red and blue in the image, respectively. The green image indicates the nucleus. *E*, *H*, and *K*: Intracellular distribution of GK. *F*, *I*, and *L*: Intracellular ditribution of GKRP. *D*, *E*, and *F* = control group; *G*, *H*, and *I* = paired-fed group; *J*, *K*, and *L* = T-1095 treatment group. Values are means ± SE of the ratio of nuclear to cytoplasmic immunofluorescence activity of GK and GKRP for five rats in each group. ND, not determined.

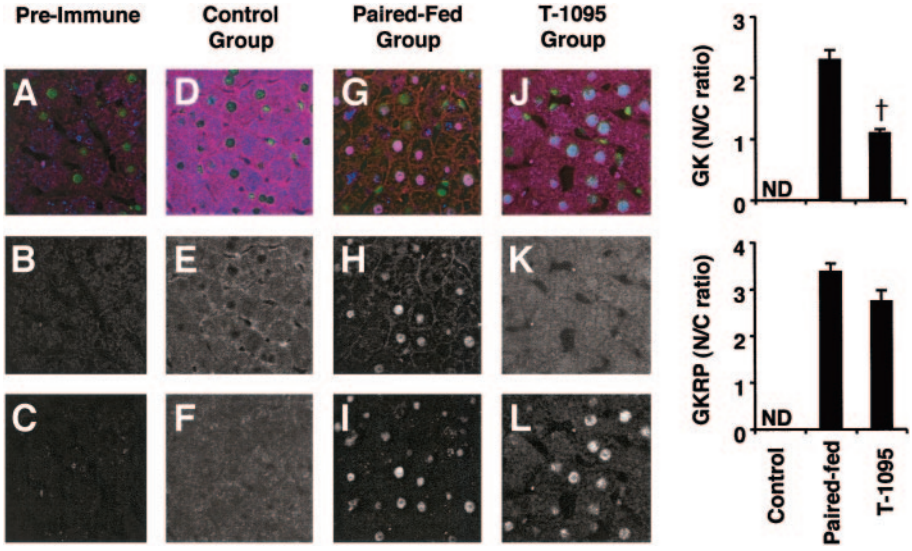

FIG. 6. The intracellular distribution of GK and GKRP in hepatocytes at the end of each hyperinsulinemic-hyperglycemic clamp study. *A*, *D*, *G*, and *J*: Immunofluorescence stainings for GK and GKRP are revealed as red and blue in the image, respectively. The green image indicates the nucleus. *E*, *H*, and *K*: Intracellular distribution of GK. *F*, *I*, and *L*: Intracellular distribution of GKRP. *D*, *E*, and *F* = control group; *G*, *H*, and *I* = paired-fed group; *J*, *K*, and *L* = T-1095 treatment group. Values are means ± SE of the ratio of nuclear to cytoplasmic immunofluorescence activity of GK and GKRP for five rats in each group. †*P* < 0.05 vs. the paired-fed group. ND, not determined.

T-1095 group compared with the other groups (Table 1). The incorporation of plasma glucose via the direct pathway and PEP into hepatic glycogen during the basal study was low and similar among the three groups (Table 1). At the end of the clamp study, in the paired-fed group, hepatic glycogen synthesized from plasma glucose via the direct pathway and from PEP was slightly higher compared with that at the end of the basal study in the identical group. In the T-1095 treatment group, hepatic glycogen synthesized from plasma glucose via the direct pathway was increased sevenfold compared with that at the end of the basal study in the identical group. The ratio of [2-^{3}H] to [3-^{3}H] glucose incorporation into glycogen was increased twofold. However, the incorporation of PEP into hepatic glycogen was increased by only 50% but was not statistically different from the paired-fed group. Despite much higher glycogen synthesis (54.2 μmol/g liver as the sum of the incorporation of plasma glucose and PEP) in the clamp study compared with that (13.6 μmol/g liver) in the basal study in the T-1095 group, hepatic glycogen contents were not significantly different between both studies. We do not have any substantial evidence to explain this discrepancy. It is possible that the T-1095–treated animals selected for the clamp study, compared with the animals selected for the basal study, had relatively lower hepatic glycogen content before the treatment with T-1095 or larger decrement in glycogen content by T-1095 treatment.

Glycogen content (μmol glucose/g muscle) in skeletal muscle at the end of the basal (25.7 ± 2.1 in the control, 25.1 ± 1.8 in the paired-fed, and 25.2 ± 1.5 in T-1095 groups) and clamp studies (26.1 ± 1.9 in the control, 25.9 ± 1.3 in the paired-fed, and 27.8 ± 2.4 in the T-1095 groups) was not different among the three groups. In response to the clamp, the amount of glucose incorporated into muscle glycogen (μmol glucose/g muscle) was increased similarly in the paired-fed (2.95 ± 0.85) and T-1095 treatment groups (3.01 ± 1.02) but not in the control group (0.8 ± 0.21), compared with that under the basal condition (0.62 ± 0.16 in the control, 1.04 ± 0.24 in the paired-fed, and 0.44 ± 0.09 in the T-1095 groups).

Activities of GK, G6Pase, glycogen synthase, and phosphorylase and expressions of GK and GKRP in the liver. The catalytic activities of hexokinase (GK measured with glucose concentrations at both 8 and 100 mmol/l, which give near–half-maximal and maximal velocities of the enzyme, respectively, in normal GK [29]) and total G6Pase and the expression levels of GK and GKRP proteins measured by Western blot analysis were not significantly different among the three groups under either basal or clamp conditions (data not shown). The activities of glycogen synthase I and phosphorylase a and the total activities of the synthase and phosphorylase were not significantly different among the three groups under the basal condition and were not different between basal and clamp conditions in the control and paired-fed groups under the clamp condition (data not shown). In the T-1095 group, on the other hand, the synthase I activity (0.20 ± 0.04 units/g liver) and the ratio of I to total activity (17 ± 3%) were significantly higher, and, in contrast, the activity of phosphorylase a (8.52 ± 3.8 units/g liver) was significantly lower compared with that in the identical group (0.09 ± 0.01, 8.8 ± 0.6, and 18.6 ± 2.6, respectively) under the basal condition and the other groups (0.11 ± 0.03, 7.0 ± 0.9, and 18.5 ± 3.2, respectively, in the control group and 0.1 ± 0.01, 7.9 ± 1.1, and 17.5 ± 2.6, respectively, in the paired-fed group) under the clamp condition.

Intracellular distribution of GK and GKRP. Under basal conditions (Fig. 6), GK and GKRP immunoreactivities were localized predominantly to the nucleus in the paired-fed (N/C ratio; 2.72 ± 0.37 and 3.41 ± 0.43, respectively) and T-1095 treatment group (N/C ratio; 2.78 ± 0.37 and 2.93 ± 0.24, respectively). The fractions of GK- and GKRP-positive cells were not different between the paired-fed (64 ± 8 and 68 ± 6%, respectively) and the T-1095 (65 ± 6 and 67 ± 7%, respectively) groups. Under the clamp condition (Fig. 7), GK and GKRP immunoreactivities were concentrated in the nucleus (N/C ratio; 2.40 ± 0.20 and 3.33 ± 0.23, respectively) in the paired-fed group. However, in the T-1095 treatment group, GK immunoreactivity was increased in the cytoplasmic compartment and

decreased in the nuclear compartment (N/C ratio; 1.09 ± 0.07). GKRP immunoreactivity was detected in the nucleus (N/C ratio; 2.74 ± 0.24). The fraction of GKRP-positive cells was not different from that under basal condition in both groups (69 ± 8 in the paired-fed and 65 ± 6% in the T1095 groups). In the control group, on the other hand, GK and GKRP immunoreactivities were not detected in the nucleus in the selected area (Figs. 6 and 7) and even in any area of the hepatic lobules by examination of broad sections of the liver (data not shown) under both the basal and clamp conditions.

DISCUSSION

The present study demonstrated that short-term normalization of hyperglycemia and/or decreased retention of glucose in the body improves impaired suppression of hepatic glucose production and ameliorates the defect in HGU in response to elevated glucose and/or insulin in ZDF rats. Furthermore, the improvement was associated with normalization of a defect in the dissociation of GK from GKRP and GK translocation from the nucleus to the cytoplasm.

Hepatic glucose fluxes observed in ZDF rats replicate those observed in patients with diabetes. The characteristics in hepatic glucose metabolism seen in obese diabetes in human are slightly increased NHGP (2,3), increased GNG (2,3), increased GC (30–32), and decreased contribution of glycogenolysis to HGP (33,34). In 14-week-old ZDF rats without any treatment (the control group), in the presence of hyperglycemia and hyperinsulinemia, glucose was continuously produced endogenously. Approximately 50% of G6Pase flux was due to GC, while GC values of 20% of G6Pase flux were measured in 6-h– (21) and 24-h–starved normal rats (35). GNG from PEP was responsible for 46% of G6Pase flux and 90% of EGP, suggesting that HGP was due mainly to GNG through PEP. Conversely, other pathways, including GNG from glycerol and glycogenolysis, were responsible for <10% of EGP, indicating a very small contribution of net glycogenolysis to NHGP despite the presence of high glycogen content in the liver. Therefore, the hepatic glucose fluxes seen in this model replicated those reported in individuals with obese diabetes.

Impaired hepatic glucose fluxes seen in ZDF rats were improved by restoration of near-normal glycemia. In the T-1095–treated group, in comparison with other two groups that were not treated with T-1095, EGP was slightly lower and G6Pase flux and GC were markedly decreased despite lower plasma glucose and insulin levels. Hepatic glycogen content was significantly lower, and the contribution of other pathways, including GNG from glycerol and glycogenolysis, to EGP was higher (22%), implying an increased contribution of net glycogenolysis to EGP. These results indicated that basal hepatic glucose flux was partially improved.

When plasma insulin and glucose levels were elevated to the level of that seen in the control group, EGP was suppressed completely in the T-1095 treatment group, while sustained EGP was observed in the control group, indicating that the ability of insulin and/or glucose to suppress NHGP was restored by the treatment with T-1095. Compared with the control group, GC (glucose→G-6-P→glucose) and the incorporation of plasma glucose into hepatic glycogen via the direct pathway (glucose→G-6-P→G-1-P→UDP-G→glycogen) were 2.5 and 5 times higher, respectively (Table 1). The higher fractional contribution of plasma glucose to UDP-G flux suggested that the flux of glucose to the G-6-P pool was higher, and, therefore, the higher rates of GC and the incorporation of plasma glucose into glycogen were not due simply to the increased activities of G6Pase and glycogen synthase, respectively. The ratio of [2-^{3}H] to [3-^{3}H] glucose incorporation into hepatic glycogen was twofold higher in the T-1095–treated rats. A study using cultured hepatocytes showed that increased GK activity was associated with increased glycogen synthesis via the direct pathway and the ratio of [2-^{3}H] to [3-^{3}H] incorporation into glycogen (36). Taken together, these data suggest that treatment with T-1095 dramatically improved the responsiveness of GK flux to elevated plasma glucose and/or insulin in ZDF rats, although the methods used did not allow direct assessment of HGU and GK flux.

Treatment with T-1095, given as food admixtures, restored near-normal postabsorptive plasma glucose levels immediately (Fig. 1) and lessened postprandial glycemia (Fig. 2) without alteration of plasma levels of insulin, glucagon, FFAs, blood lactate, and alanine that affect hepatic glucose metabolism (Figs. 1 and 2). T-1095 treatment caused a decrease in food intake during the feeding period, which was in agreement with a previous report (10). Moreover, it was reported (37) that a decrease in total calorie intake may ameliorate insulin resistance. However, in the paired-fed group postabsorptive hyperglycemia (22 mmol/l) and marked postprandial hyperglycemia remained, and basal hepatic glucose fluxes were similar to those in the control group. The hyperglycemic-hyperinsulinemic–induced suppression of EGP and HGU were still impaired and were not statistically different from the control group. Therefore, improvement of hepatic glucose metabolism by treatment with T-1095 may be associated with the restoration of near normoglycemia and/or decreased retention of glucose in the body but did not result from decreased total calorie intake.

T-1095 treatment–induced improvement of basal glucose fluxes was not associated with alteration of the expression and the distribution of GK and GKRP. The mechanism responsible for increased GC in diabetes remains unknown. Increased GC must be associated with increased rates of either or both phosphorylation of glucose and dephosphorylation of G-6-P. Barzilai et al. (38) reported that decrease in GK activity by glucosamine infusion can decrease GC in the presence of hyperglycemia in normal rats, suggesting that changed GK activity affects GC. T-1095 treatment decreased the fractional contribution of plasma glucose to UDP-G flux by 30% (Table 1), implying decreased glucose phosphorylation. However, the decreased GC resulting from T-1095 treatment was not related to any alteration in total GK activity or the ratio of GK to GKRP expression (Fig. 5) or the intracellular localization of GK and GKRP (Fig. 6). Although another subtype of hexokinase exists in the liver (39), the activity of this enzyme was not decreased by T-1095 treatment (Fig. 5). Therefore, it is unlikely that the decrease in GC resulting from T-1095 treatment was associated with alteration of hepatic GK and hexokinase activity. However, decreased plasma glucose levels (Fig. 3) might have contributed to decreased glucose phosphorylation via the decrease in mass action. On the other hand, increased GC has been observed in humans and animals not only with type 2 diabetes (30–32) but also with type 1 diabetes (40,41), where hepatic GK activity is often mark-

edly decreased, suggesting contribution of factor(s) other than GK activity to increased GC. Changes in the disposal rate of G-6-P formed from plasma glucose could affect GC without altered GK flux. Henly et al. (41) reported that suppression of glycolysis is associated with an increase in GC in hepatocytes from streptozotocin-induced diabetic rats. While the incorporation of plasma glucose into glycogen under basal conditions was not increased by the T-1095 treatment (Table 1), it remains possible that increased glycolysis decreased GC in the T-1095–treated group. Furthermore, the degree of decrease in GC by the T-1095 treatment exceeded that of the fractional contribution of plasma glucose to UDP-G flux, and decreased GC was accompanied by a decrease in both G6Pase flux and gluconeogenic flux without changes in G-6-P content (Table 1), indicating decreased G6Pase activity. Although it has been reported that increased glucose concentration stimulates G6Pase expression in the liver (42), total G6Pase activity was not altered by T-1095 treatment (Fig. 5). Recently, it was reported that G6Pase activity is allosterically regulated by insulin signaling (43). Therefore, it is possible that the ability of insulin to inhibit G6Pase allosterically was improved by T-1095 treatment.

Improved HGU is associated with normalized regulation of GK by GKRP. Changes in total GK activity (36) and the molar ratio of GK to GKRP protein (44) have a large impact on HGU and hepatic glycogen synthesis via the direct pathway. However, this was not the case in the present study. T-1095 treatment did not alter the amount of GK protein and the catalytic function of the enzyme present (Fig. 5). Because the expression of GKRP was not different among the three groups, the molar ratios of GK to GKRP were not altered by the T-1095 treatment (Fig. 5). On the other hand, the dissociation of GK from GKRP in the nuclear compartment and subsequent translocation of GK into the cytosolic compartment seem to be critical for increasing glucose phosphorylation in response to elevated glucose and insulin. When GK was coexpressed with GKRP in HeLa cells or human embryonic kidney cells, where normal GK accumulated with GKRP in the nucleus (45,46), an engineered mutant form of GK with reduced affinity for GKRP did not concentrate in the nucleus even at low glucose levels (46,47). Mice that were mutant null for GKRP exhibited cytoplasmic localization of GK even at low blood glucose levels (48,49). These findings indicated that the binding of GK to GKRP is essential for localizing GK in the nucleus. In studies using cultured hepatocytes, monosaccharides such as fructose and sorbitol, precursors of fructose-1-phosphate that stimulates the dissociation of GK from GKRP (19), always caused translocation of GK, but not GKRP, from the nucleus (18) and increased glucose phosphorylation and glycogen synthesis from glucose (18). We showed that small amounts of fructose increased NHGU and glycogen synthesis from plasma glucose in conscious normal dogs (50,51). Furthermore, GK translocation was brought about by elevation of plasma insulin and glucose concentration within the physiological range, which are major stimulators of HGU, in normal conscious rats (20,21). These data suggested that the dissociation of GK from GKRP in the nucleus and subsequent GK translocation to the cytoplasmic compartment mediated by glucose and/or insulin play a critical role in acute stimulation of hepatic glucose phosphorylation and NHGU in the liver of normal individuals. In the present study, regardless of the presence of marked hyperglycemia and hyperinsulinemia, GK and GKRP were colocalized to the same intracellular compartment, in the cytoplasm of the control group and in the nucleus of the paired-fed group (Figs. 6 and 7). Conversely, T-1095 treatment restored the normal distribution of GK and GKRP in the nucleus and glucose-induced translocation of GK and separated localization of GK and GKRP (Figs. 6 and 7), which suggested that improvement in HGU in response to elevated plasma glucose and/or insulin by T-1095 treatment was associated with normalized regulation of GK by GKRP. It is likely that chronic hyperglycemia induces abnormal distribution of GKRP and the impairment of glucose and/or insulin-induced dissociation of GK from GKRP and of GK translocation.

Since GK and GKRP are not expressed homogeneously in all the parenchymal cells in the liver and the magnitude of the expression varies among the cells that express both proteins, the evaluation of the subcellular localization and translocation of these proteins in the present study was accompanied by a certain assumption. We selected cells as GK and GKRP positive with the index of detectable GKRP immunofluorescence in the nucleus based on following reasons. *1*) If these proteins are originally located in the cytoplasm or exported in large part from the nucleus in response to a stimuli, it is technically difficult to identify a GK/GKRP-positive cell because the immunoreactivity might be very low due to the extensive dilution in the space of the cytoplasm, which is ~10 times larger than the space of the nucleus in hepatocytes, *2*) GK is always coexpressed with GKRP, and *3*) GKRP stays in the nucleus normally regardless of the nutritional state, while GK translocates in response to changes in nutritional state (20,21). It must be acknowledged, however, that with the adoption of this index, it is possible that the ratio of nuclear to cytoplasmic GK and GKRP overestimates the nuclear localization of these proteins and underestimates GK translocation. This is because the cells would not be selected if GKRP was localized in the cytoplasm or largely exported from the nucleus. This was evident in the control group in the present study. While Western blot analysis showed the presence of similar amounts of GK and GKRP proteins in the livers in the control group with that in the other groups, immunoreactivities of both GK and GKRP were not detected in the nucleus in all hepatocytes, indicating that these proteins were localized in the cytoplasm. On the other hand, in the paired-fed and T-1095 groups, GK and GKRP were detected in the nucleus in ~65% of hepatocytes under the basal condition and GK coexisted with GKRP in the nucleus. Under the clamp condition, the population of GKRP-positive cells was also similar between the paired-fed (69%) and the T1095 (65%) group. The population of hepatocytes with a GKRP-positive nucleus in these groups was also similar to that in 6-h–fasted normal rat liver (67 ± 5%, $n = 5$). It is likely, therefore, that normal localization of GKRP in the nucleus was restored in the paired-fed and T-1095 groups, although the reason remains unknown and although it could not be guaranteed that GKRP was localized in the nucleus in all GK- and GKRP-positive cells in these groups.

Improved hepatic glycogen synthesis in response to elevated plasma glucose. The response of the liver to a glucose load involves the sequential inactivation of glycogen phosphorylase and activation of glycogen synthase in most (52–54) but not all (55) studies. A major part of the glucose taken up by the liver is normally stored as glycogen (56). Therefore, the linkage of increased glycogenesis with increased glucose phosphorylation may be

critical in HGU in response to elevated glucose. Chronic normalization of hyperglycemia by T-1095 treatment improved total glycogen synthesis in response to elevated plasma glucose (Table 1), and the improvement was accompanied by the activation of glycogen synthase and inactivation of glycogen phosphorylase. Activation of glycogen synthase in hepatocytes by a glucose load has been attributed to an increased content of the intermediate (G-6-P), a potent activator of glycogen synthase (57), via increased glucose phosphorylation (58). This is not the case in the current study because there were no differences in liver G-6-P content among the three groups during the glucose clamp (Table 1). Glucose per se is also a strong regulator in activating glycogen synthase and in inhibiting glycogen phosphorylase in the liver (59). Binding of glucose to phosphorylase a causes a conformational change that renders the enzyme a better substrate for dephosphorylation by protein phosphatase-1. Glucose thus favors the conversion of phosphorylase a to phosphorylase b. Because phosphorylase a is a potent inhibitor of glycogen synthase phosphatase, the decrease in phosphorylase a relieves the inhibition of synthase phosphatase. Glucose- and/or insulin-induced activation of glycogen synthase in the liver is markedly impaired in ZDF rats (21). Therefore, it is likely that impaired regulation of glycogen phosphorylase and synthase activities by glucose is improved in ZDF rats by the treatment with T-1095. The mechanism responsible for impaired regulation of glycogen phosphorylase and synthase activities by glucose in this model of obese type 2 diabetes, and the improvement by chronic restoration of hyperglycemia remains unknown.

ZDF rats have two to three times higher glycogen content compared with the lean littermates (21). The T-1095 treatment decreased hepatic glycogen content by 30% (Table 1). The higher glycogen content in ZDF rats was accompanied by a much lower glycogenolytic flux (Table 1). The contribution of glycogenolysis to EGP was extremely low (<4%; Table 1) in ZDF rats compared with that (~50%) reported in normal rats using a similar analytical method (60). Indeed, hepatic glycogen content in 14-week-old ZDF rats was not decreased by longer fasting (296 ± 27 in 24-h fasted [$n = 5$] vs. 304 ± 48 in 6-h fasted [$n = 6$]). Increased hepatic glycogen content and decreased net glycogenolysis have been reported in obese humans (34) and obese rodent models (61,62). An increased plasma FFA level has been reported to increase hepatic glycogen content (63) and to decrease hepatic glycogenolysis (63,64) in healthy humans. Glucose at concentrations that are within the physiological range (8–20 mmol/l) strongly inhibits hepatic glycogen phosphorylase a activity in a dose-dependent manner via an allosteric mechanism (65). An increased blood glucose level inhibits net hepatic glycogenolysis by decreasing glycogen phosphorylase flux in overnight-fasted subjects (14). Therefore, hyperlipidemia and hyperglycemia might contribute to maintain hepatic glycogen content at higher-than-normal levels. The T-1095 treatment reduced plasma glucose levels in both the postabsorptive (Fig. 1) and postprandial (Fig. 2) states, although the treatment did not alter plasma FFA levels (Figs. 1 and 2). The fractional contribution of sources (including glycogen) other than plasma glucose and PEP to UDP-G flux tended to be higher in the T-1095 group (Table 1). It is possible that T-1095 treatment decreases hepatic glycogen content by partially releasing the hyperglycemia-mediated inhibition of glycogenolysis in the ZDF rats.

In summary, the results from the present study suggested that chronic hyperglycemia impairs glucose- and/or insulin-induced dissociation of GK from GKRP that is responsible, at least partly, for the impaired response of hepatic glucose flux to elevated glucose and/or insulin. It has been reported in individuals with type 2 diabetes that fructose decreases the glucose and insulin response to an oral glucose tolerance test (66) and that the ability of hyperglycemia per se to suppress hepatic glucose production was nearly normalized by the addition of a catalytic amount of fructose (67). Thus, it is possible that chronic hyperglycemia-induced impairment of short-term activation of GK via dissociation of this enzyme from GKRP could be one cause of defective HGU in subjects with obese type 2 diabetes. The mechanism by which glucose- and/or insulin-induced dissociation of GK from GKRP and subsequent translocation in the liver are impaired by chronic hyperglycemia is a subject for further study.

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