Defect in glucokinase translocation in Zucker diabetic fatty rats

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Fujimoto, Yuka, E. Patrick Donahue, and Masakazu Shiota. Defect in glucokinase translocation in Zucker diabetic fatty rats. Am J Physiol Endocrinol Metab 287: E414–E423, 2004. First published May 11, 2004; 10.1152/ajpendo.00575.2003.—Hepatic glucose fluxes and intracellular movement of glucokinase (GK) in response to increased plasma glucose and insulin were examined in 10-wk-old, 6-h-fasted, conscious Zucker diabetic fatty (ZDF) rats and lean littermates. Under basal conditions, plasma glucose (mmol/l) and glucose turnover rate (GTR; mmol-kg⁻¹-min⁻¹) were slightly higher in ZDF (8.4 ± 0.3 and 53 ± 7, respectively) than in lean rats (6.2 ± 0.2 and 45 ± 4, respectively), whereas plasma insulin (pmol/l) was higher in ZDF (1,800 ± 350) than in lean rats (150 ± 14). The ratio of hepatic uridine 5'-diphosphate-glucose 3H specific activity to plasma glucose 3H specific activity ([3H]UDP-G/3H[G]; %), total hepatic glucose output (μmol-kg⁻¹-min⁻¹), and hepatic glucose cycling (μmol-kg⁻¹-min⁻¹) were higher in ZDF (35 ± 5, 87 ± 16, and 33 ± 10, respectively) compared with lean rats (18 ± 3, 56 ± 6, and 11 ± 2, respectively). [3H]glucose incorporation into glycogen (μmol glucose/g liver) was similar in lean (1.0 ± 0.7) and ZDF (1.6 ± 0.8) rats. GK was predominantly located in the nucleus in both rats. With elevated plasma glucose and insulin, GTR (μmol-kg⁻¹-min⁻¹), [3H]UDP-G/3H[G] (%), and [3H]glucose incorporation into glycogen (μmol glucose/g liver) were markedly higher in lean (191 ± 22, 62 ± 3, and 5.0 ± 1.4, respectively) but similar in ZDF rats (100 ± 6, 37 ± 3, and 1.4 ± 0.4, respectively) compared with basal conditions. GK translocation from the nucleus to the cytoplasm occurred in lean but not in ZDF rats. The unresponsiveness of hepatic glucose flux to the rise in plasma glucose and insulin seen in prediabetic ZDF rats was associated with impaired GK translocation.

PATIENTS WITH TYPE 2 DIABETES exhibit preprandial hyperglycemia and excessive postprandial hyperglycemia. The preprandial hyperglycemia is due to impaired suppression of endogenous glucose production in response to increased plasma glucose and/or insulin as well as impaired glucose uptake in peripheral tissues (19, 41, 42). In addition to these impairments, a defect of splanchnic glucose uptake has been reported to contribute to the occurrence of excessive postprandial hyperglycemia (9, 10, 24). Changes in plasma glucose and insulin are major factors regulating net hepatic glucose flux (8, 52). The 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 (G-6-Pase). In studies using normal rats, glucose-induced suppression of net hepatic glucose production (HGP) was associated with increased glucose phosphorylation (53), and intact GK activity was required for the normal suppression of HGP by hyperglycemia (7). Basu et al. (9, 10) showed that lower net splanchnic glucose uptake during a hyperglycemic hyperinsulinemic clamp in type 2 diabetic subjects was associated with a proportionate decrease in both the flux through the uridine 5'-diphosphate (UDP)-glucose pool and the percentage of contribution of extracellular glucose to glycogen synthesis by the direct pathway (glucose → G-6-P → G-1-P → UDP-glucose → glycogen) compared with nondiabetic subjects. Mevorach et al. (42) reported a lack of increasing glucose cycling (GC) in the presence of unchanged flux through G-6-Pase in the face of a doubling in circulating glucose concentration. It is likely, therefore, that impaired suppression of net HGP and a defect in hepatic glucose uptake in response to increased plasma glucose and/or insulin seen in type 2 diabetes result, at least partly, from the failure of the increase in the plasma glucose concentration to enhance the flux through GK.

GK activity is known to be regulated by changes in the amount of enzyme present due to alteration in its gene transcription. However, the amount of GK in the liver is not always decreased in subjects (11) and animals (34) with obese type 2 diabetes. Recently, GK activity has also been shown to be regulated by its 68-kDa regulatory protein (GKRP) in the liver (63). GKRP binds to GK and allosterically inhibits the enzyme by decreasing the apparent affinity for glucose (63). The inhibition of GK activity by GKRP is due to competitive inhibition of glucose binding to GK (63) and is released by fructose 1-phosphate (F-1-P), which binds to GKRP and thereby decreases its affinity for GK (16). The regulation of GK by GKRP seems to be associated with a change in intracellular distribution of GK. In studies using cultured hepatocytes (13, 60), GK and GKRP were predominantly located in the nucleus when hepatocytes were cultured with 5.5 mM glucose. However, catalytic amounts of fructose and sorbitol, a precursor of F-1-P, caused GK export from the nucleus (13, 37, 62). These precursors also markedly increased the rate of glucose phosphorylation in cultured hepatocytes (3, 16, 62). We showed that intraperitoneal (57) or intraduodenal (58) administration of a small amount of fructose markedly increased net hepatic glucose uptake in conscious dogs, suggesting that the activation of GK by dissociation from GKRP and subsequent translocation from the nucleus to the cytoplasm has a large impact on the regulation of hepatic glucose flux.

Changes in plasma glucose and/or insulin concentrations have been shown to be major factors in regulating net hepatic glucose flux by suppressing glucose production and by stimulating glucose uptake (52, 53). Studies using cultured hepatocytes have repeatedly shown that glucose per se is a potent stimulator of GK translocation (2, 13, 60, 62), whereas the results regarding the effect of insulin are controversial (3, 13, 61). We recently reported (15) that increases in plasma glucose

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and/or insulin levels within the physiological range cause rapid GK translocation in conscious normal rats. These results from in vitro and in vivo studies led us to hypothesize that impaired translocation of GK is responsible for a defect in hepatic glucose uptake seen in obese type 2 diabetes. To test this hypothesis, we compared hepatic glucose flux and intracellular movement of GK in response to combined increase in plasma glucose and insulin between 10-wk-old Zucker diabetic fatty (ZDF) rats, which are at early stage of the development of diabetes, and their lean littermates.

**RESEARCH DESIGN AND METHODS**

**Animals and surgical procedure.** Male ZDF (ZDF/Gmi-fa/fa) rats and their littermates (ZDF/Gmi-+/fa) were purchased from Genetic Models (Indianapolis, IN) when they were 6 wk old. Rats were fed with no. 5008 Formulab Diet (Purina Mills, St. Louis, MO) and were given water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle. Surgery was performed at 8 wk of age, 2 wk before the experiment, as described previously (15). Rats were anesthetized with a single intraperitoneal injection of pentobarbital sodium (42.5 mg/kg body wt). After a midline laparotomy, a sterile silicone rubber catheter (0.51 mm ID, 0.94 mm OD) was placed in an ileal vein. The left common carotid artery and the right external jugular vein were cannulated with sterile silicone rubber catheters (0.51 mm ID, 0.94 mm OD, and 0.64 mm ID, 1.19 mm OD, respectively). The free ends of these catheters were subcutaneously passed to the back of the neck where they were fixed. These catheters were filled with heparinized saline solution (200 U heparin/ml saline) and closed with metal plugs. The catheters were placed only in the left common carotid artery and the right jugular vein on some of the lean rats, which were used as blood donors. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of both the United States Department of Agriculture and the National Institutes of Health, and all protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

**Experimental design.** The animals were fasted for 6 h before the study. Each study consisted of a 2-h tracer equilibration period (~180 to ~60 min), a 1-h control period (~60 to 0 min), and a 1-h test period (0 to 60 min). At ~180 min, [3-3H]glucose was given at 60 μCi in a bolus through the jugular vein catheter and a continuous infusion at 0.6 μCi/min was begun. During the test period, in a basal group of both lean and ZDF rats, the animals were kept without additional treatment. In the clamp group, somatostatin was infused through the jugular vein catheter at 2 μg·kg⁻¹·min⁻¹ to inhibit endogeneous insulin and glucagon secretion. Insulin and glucagon were infused into the hepatic portal system through the ileal vein catheter at 8.4 mU·kg⁻¹·min⁻¹ and 2.6 ng·kg⁻¹·min⁻¹, respectively. Plasma glucose levels were kept at 15 mmol/l by infusion of 50% glucose solution at a variable rate. The infusion rates of [3-3H]glucose were increased to 1.2 and 2.4 μCi/min in ZDF and lean rats, respectively, to maintain the specific activity in plasma glucose. Blood samples were taken from the arterial catheter. Blood glucose levels were monitored using HemoCue (HemoCue, Mission Viejo, CA), a glucose analyzer. At each sampling time, the whole blood from the donor rat was given back to the test animal during the study. At the end of experiment, the animal was anesthetized with an intravenous infusion of pentobarbital sodium (40 mg/kg), and a laparotomy was immediately performed. The median lobe of the liver was excised and dropped into ice-cold phosphate-buffered saline (PBS) for immuno-histochemical analysis. The left lobe of the liver and skeletal muscle (vastus lateralis) were frozen in situ using Wollenberg tongs precooled in liquid nitrogen. This procedure took less than 20 s from the point of successful anesthesta.

**Immunostaining and Western blot analysis for GK and GKR.** An anti-glutathione-S-transferase (GST)-GK fusion protein and an anti-GST-GKR fusion protein were produced in a sheep and a rabbit, respectively. The entire coding of GK and GKR cDNA obtained from rat liver RNA by RT-PCR was cloned and inserted in a pGEX-2T (Pharmacia) vector and expressed as a GST fusion protein in Escherichia coli BL21. The fusion proteins were purified by GST-agarose affinity chromatography and used as an immunogen in sheep and rabbit, respectively. The immunostaining of GK and GKR and Western blot analyses of these proteins were performed as reported previously (15). The median lobe of the liver was quickly excised, rinsed in PBS, and diced into cubes. The cubes were immediately immersed in 4% paraformaldehyde-PBS on ice and fixed for 3 h. After a wash and conventional paraffin embedding, two serial 4-μm sections were mounted on the same slide glass. After being deparaffinized, hydrated, and blocked in 10% normal donkey serum-PBS for several hours, an equilibrium mixture of sheep anti-rat GST-GK serum and rabbit anti-rat GST-GKR serum (diluted 1:2,000 in 5% normal donkey serum plus 0.1% Triton X-100-PBS) was dropped onto one of the sections. An equilibrium mixture of premixed sheep and rabbit serum (diluted 1:1,000 in 5% normal donkey serum plus 0.1% Triton X-100-PBS) was dropped onto the other section. Slides were kept in a humid chamber for 12 h at 4°C. After 5 min washes in 0.1% Triton X-100-PBS, slides were incubated with Cy3-conjugated donkey anti-sheep IgG (diluted 1:1,000), Cy5-conjugated donkey anti-rabbit IgG (diluted 1:1,000), and Cy5-conjugated goat anti-rabbit IgG (diluted 1:1,000) in 5% normal donkey serum-PBS for 1 h at room temperature in the dark. After four 5-min washes in PBS, sections were mounted with Aqua Poly/Mount. As a control, standard sections, which were sampled from 6-h-fasted rats under the condition of euglycemia and euinsulinemia, were stained at the same time as the sample sections. Cy3-conjugated donkey anti-sheep IgG, Cy5-conjugated anti-rabbit IgG, YoPro-1, and normal donkey serum were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA).

Western blot analyses were performed using the same sheep anti-rat GST-GK serum (diluted 1:5,000) and rabbit anti-rat GST-GKR serum (diluted 1:5,000) as described in Ref. 36.

**Metabolites in blood and tissue.** Plasma glucose, 3H radioactivity and glucose concentrations of the supernatant of deproteinized plasma were determined by the method of De Bodo et al. (17). Blood concentrations of lactate (49), glycerol (66), and alanine (67) were determined according to the enzymatic analytical methods in blood samples deproteinized with perchorlic acid (3%). Plasma triglyceride (TG) and free fatty acid (FFA) concentrations were determined using a kit (Sigma Diagnostics, St. Louis, MO) and the Wako nonesterified fatty acid C test kit (Wako, Osaka, Japan), respectively. The concentrations of G-6-P in the liver were determined using the method described by Michal (43). The glycogen concentrations in liver and skeletal muscle were determined by the amyloglucosidase method (14). 3H counts in glycogen were determined after liquid scintillation counting of the processed samples.

The concentrations of UDP-glucose and UDP-galactose and 3H radioactivity in these metabolites in the liver were obtained through two sequential chromatographic separations by a modification of the method of Giaccari and Rossetti (29). Liver samples (~500 mg) were homogenized in 1 ml of 0.6 mol/l ice-cold perchloric acid. The homogenate was kept at 0°C for 5 min and then centrifuged at 2,850 g at 0°C for 10 min. The supernatants were separated and diluted with 7 ml of 10 mmol/l potassium phosphate (pH 6.5). Solid-phase extraction was performed on a 3-ml SPE Supelclean LC-SAX strong anion-exchange cartridge (Supelco, Bellefonte, PA), using an aqueous potassium dihydrogenphosphate solution of increasing molality: 10 mmol/l (5 ml), 50 mmol/l (1.5 ml), and 100 mmol/l (1.5 ml) for UDP-glucose (pH 6.5). The fraction of 100 mM containing ≥96% of UDP-glucose and UDP-galactose was applied to the HPLC system consisting of two 510 pumps, a 680 programmable gradient controller, a 712 injector, and a 481 turnable absorbance detector (all from Waters Chromatography Division, Millipore, Mil-
ford, MA). Chromatography was carried out on two HPLC LC-18-T Supelcosil strong anion-exchange columns (250 × 4.6 mm ID) in series from Supelco. Peak areas were integrated on an HP-3396 recorder (Hewlett-Packard, Avondale, PA). Fractions were collected with a Retriever IV fraction collector (ISCO, Lincoln, NE). The isocratic system used an aqueous 100 mmol/l potassium dihydrogen phosphate solution containing 5 mmol/l tetrabutylammonium hydroxysulfate solution prepared with distilled, deionized water adjusted to pH 6.0 with 200 mmol/l orthophosphoric acid and was refiltered and degassed with a 0.45-μm filter. The flow rate was 1.0 ml/min in all experiments, and the temperature was 35°C. Calibration was measured at 262 nm at 1.0 absorbance unit full scale.

**Plasma hormones.** The concentrations of plasma insulin and glucagon were determined by radioimmunoassay using rat insulin and glucagon radioimmunoassay kits, respectively (Linco Research, St. Charles, MO).

**Enzyme activities.** For GK, hexokinase (HK), and G-6-Pase activities, freeze-clamped liver was homogenized in 50 mmol/l HEPES, 100 mmol/l KCl, 1 mmol/l EDTA, 5 mM MgCl₂, and 2.5 mmol/l dithioerythritol (8). GK and G-6-Pase activities were measured in the supernatant and sedimentary fractions, respectively, after centrifugation. Glycogen phosphorylase activities in the liver were measured using the method described by Golden et al. (31).

**Glycogen synthesis.** Glycogen turnover rate (GTR) was calculated as the ratio of the rate of infusion of [3-3H]glucose (dpm/min) and the steady-state plasma [3H]glucose specific activity (SA; dpm/μmol) according to the method of De Bodo et al. (17).

HGP, which is taken to reflect the balance of the in vivo fluxes through GK and G-6-Pase, was calculated as the difference between the tracer-derived rate of appearance and the infusion rate of glucose, assuming that HGP is equivalent to the endogenous glucose production. Total glucose output (TGO) is defined as the total in vivo flux through G-6-Pase. 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. According to the method of Giacciari and Rossetti (30), the percentage of the hepatic G-6-P pool, directly derived from plasma glucose, was calculated as the ratio of [3H]UDP-glucose to plasma [3H]glucose SAs. This ratio also measures the percentage contribution of plasma glucose to TGO. The TGO is the sum of the HGP plus GC: TGO = HGP/(1 - [3H]UDP-glucose SA/[3H]glucose SA) and GC = ([3H]UDP-glucose SA/[3H]glucose SA) × TGO. To estimate the amount of extracellular glucose incorporated into glycogen via the direct pathway, 3H radioactivity incorporated into hepatic glycogen was divided by the 3H SA of plasma glucose. The rate of glycogen synthesis via the indirect pathway was calculated by using the percentage of UDP-glucose synthesized from plasma glucose [i.e., glycogen synthesis via indirect pathway = glycogen synthesis via direct pathway]/[(1 - [3H]UDP-glucose SA/[3H]glucose SA) × (1 - [3H]UDP-glucose SA/[3H]glucose SA)].

Quantitative image analysis of GK and GKRP immunofluorescence was performed using a Zeiss LSM 410 confocal laser-scanning microscope. GK and GKRP are not expressed homogenously in all the parenchymal cells in the liver. GK is expressed from periporal to perivenous areas in an increasing gradient in normal (38, 61) and ZDF rats (data not shown). Even in perivenous areas, the intensities of immunofluorescence of GK and GKRP vary among hepatocytes (data not shown). Furthermore, the extent of GK translocation in response to increased plasma glucose may differ throughout the liver lobule. To avoid intentional selection and to obtain results reflecting changes in the whole liver, three fields were randomly selected from each section. The internal He/Ne laser and external argon-krypton laser at 543, 647, and 488 nm were used to optimally excite Cy3, Cy5, and YoPro-1 fluorochromes, respectively. To identify Cy3 (GK)-positive cells, we selected cells with a higher immunofluorescence intensity of Cy5 (GKRP) in the nucleus compared with controls stained with preimmune serum, because GK is always coexpressed with GKRP. We did not detect any cells in which GK was present in the nucleus in the absence of GKRP (data not shown). 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 grayscale levels). Nuclear-to-cytoplasmic pixel density ratios of GK and GKRP were determined by digital image analysis, using Scion Image, in 10–20 cells in each field. The ratios of nuclear to cytoplasmic fluorescence of GK or GKRP were averaged for each animal, and the average value was normalized to that in the standard liver sample, stained on the same day.

**Statistical analyses.** Data are expressed as means ± SE. The significance of differences between groups of time course data was analyzed by two-way repeated-measures analysis of variance (ANOVA). The significance of differences between groups was analyzed by one-way ANOVA or Student’s t-test. Differences were considered significant when P < 0.05.

**RESULTS**

**Plasma glucose, insulin, and glucagon levels.** In the basal condition, plasma glucose levels tended to be higher in ZDF rats than in lean rats during both the control and test periods (mean values during the test period were 8.4 ± 0.3 and 6.2 ± 0.2 mmol/l, respectively; Fig. 1). Plasma insulin levels were 12-fold higher in ZDF than in lean rats (mean values during the test period were 1,819 ± 348 and 147 ± 14 pmol/l, respectively). Plasma glucagon levels were similar in ZDF and lean rats (mean values during the test period were 44 ± 1 and 42 ± 7 ng/l, respectively). In the clamp conditions, the arterial plasma glucose levels during the test period were kept at ~15
mmol/l in both ZDF and lean rats by infusing glucose peripherally. Arterial plasma insulin levels were raised to ~1.250 pmol/l in lean rats and 3,500 pmol/l in ZDF rats despite the same amount of insulin infusion, suggesting that insulin clearance may be lower in ZDF than in lean rats. Arterial glucagon levels were maintained at the basal levels.

Blood alanine, glycerol, and lactate levels and plasma FFA and TG levels. Under basal conditions, blood alanine and glyceroℓ levels were similar in ZDF and lean rats (Table 1). Blood lactate and plasma TG levels were significantly higher in ZDF rats compared with lean rats. In response to the rise in plasma glucose and insulin, blood alanine and glyceroℓ levels were not changed in either ZDF or lean rats. Blood lactate levels increased in both ZDF and lean rats. Plasma FFA and TG levels did not change in lean rats, but in ZDF rats plasma FFA levels increased and plasma TG levels decreased.

GTR and hepatic glucose fluxes. As shown in Fig. 2, in the basal condition, the GTR was similar in ZDF and lean rats (mean values during the test period were 53.4 ± 6.7 and 45.2 ± 3.7 μmol·kg⁻¹·min⁻¹, respectively). In lean rats, the combination of hyperglycemia and hyperinsulinemia caused a fourfold increase in the GTR (mean value during the test period was 191.3 ± 21.9 μmol·kg⁻¹·min⁻¹), and the GTRs were similar to glucose infusion rates (mean rate of the test period was 202.7 ± 17.6 μmol·kg⁻¹·min⁻¹), indicating that endogenous glucose production was completely suppressed. In ZDF rats, even if plasma glucose levels were raised similarly with those of the lean rats and plasma insulin levels were maintained at 2.5 times higher levels compared with the lean rats, the GTR was markedly lower compared with lean rats (mean value during the test period was 100.3 ± 6.1 μmol·kg⁻¹·min⁻¹). The glucose infusion rate (47.1 ± 13.4 μmol·kg⁻¹·min⁻¹, the average rate of the test period) of the ZDF rats was markedly lower than their turnover rate. The difference between the GTR and glucose infusion rate was similar to their GTR under the basal condition, suggesting that endogenous glucose production was not suppressed by raising plasma glucose and insulin levels.

As shown in Table 2, the percentage of UDP-glucose synthesized from plasma glucose, TGO, and the GC under basal condition were ~1.9-, 1.5-, and 3-fold higher, respectively, in ZDF than in lean rats. In response to increased plasma glucose and insulin levels, the percentage of UDP-glucose synthesized from plasma glucose increased threefold in lean rats but did not

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<th>Table 1. Arterial blood alanine, lactate, and glycerol and plasma FFA and TG levels during control and test periods in 6-h-fasted conscious lean and ZDF rats</th>
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<td><strong>Blood lactate, mmol/l</strong></td>
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<td><strong>Blood glycerol, mmol/l</strong></td>
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Values are means ± SE for 5 rats in each group. FFA, free fatty acids; TG, triglyceride; ZDF, Zucker diabetic fatty. *P < 0.05 for lean basal group vs. ZDF basal group. †P < 0.05 for basal group vs. clamp group within each animal type.
The amounts of glycogen synthesized via the direct and indirect pathways in liver was significantly higher in ZDF than in lean rats (Fig. 3). Glucose Table 2. Glucose fluxes in basal and clamp conditions in 6-h-fasted lean and ZDF rats, i.e., %UDP-glucose synthesis from glucose at end of experiment and rates of hepatic glucose production, total hepatic glucose output, and glucose cycling during test period

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<th>Lean Basal</th>
<th>ZDF Basal</th>
<th>Lean Clamp</th>
<th>ZDF Clamp</th>
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<tr>
<td>UDP-glucose synthesis from glucose, %</td>
<td>18.3±2.6</td>
<td>35.2±5.1*</td>
<td>62.1±2.6†</td>
<td>36.7±2.7</td>
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<tr>
<td>Hepatic glucose production, µmol·kg⁻¹·min⁻¹</td>
<td>45.2±3.7</td>
<td>53.4±6.7</td>
<td>−14.5±22.1†</td>
<td>51.6±9.4</td>
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<tr>
<td>Total hepatic glucose output, µmol·kg⁻¹·min⁻¹</td>
<td>55.7±5.6</td>
<td>ND</td>
<td>86.6±15.8*</td>
<td>83.7±17.4</td>
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<tr>
<td>Glucose cycling, µmol·kg⁻¹·min⁻¹</td>
<td>10.5±2.4</td>
<td>ND</td>
<td>33.3±10.3*</td>
<td>32.1±8.3</td>
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Values are means ± SE for 5 rats in each group. ND, not determined. *P < 0.05 for lean basal vs. ZDF basal group. †P < 0.05 for basal vs. clamp group within each animal type.

In the basal condition (0.79 ± 0.29 and 0.64 ± 0.13 µmol glucose/g muscle, respectively). The rise in plasma glucose and insulin markedly increased [³H]glucose incorporation into glycogen in lean rats (3.56 ± 0.76 µmol glucose/g muscle) but not in ZDF rats (1.28 ± 0.76 µmol glucose/g muscle).

G-6-P concentration in liver. The G-6-P concentration in the liver was significantly lower in ZDF rats (0.29 ± 0.07 µmol/g liver) compared with lean rats (0.51 ± 0.10 µmol/g liver) in the basal conditions (Fig. 4). In response to increased plasma glucose and insulin levels, the G-6-P concentration decreased by 50% in lean rats (0.24 ± 0.02 µmol/g liver) but did not change in ZDF rats (0.37 ± 0.02 µmol/g liver).

Intracellular distribution of GK and GKRP. In the basal condition, both GK and GKRP immunostaining were detected mainly in the nucleus in both ZDF and lean rats (Fig. 5). The ratio of nuclear to cytoplasmic GK immunoreactivities was similar in ZDF and lean rats (3.26 ± 0.28 and 3.07 ± 0.53, respectively). When plasma glucose and insulin were raised, GK, but not GKRP, immunoreactivity in the nuclear compartment decreased markedly in the lean rats. In ZDF rats, on the other hand, GK and GKRP immunoreactivities were still concentrated mainly in the nucleus. Thus, upon raising of plasma glucose and insulin, the ratio of nuclear to cytoplasmic GK immunoreactivities decreased in lean rats (1.76 ± 0.27) but not in ZDF rats (3.34 ± 0.25). These changes in GK distribution were not due to variation in the total amount of GK, because the amount of GK protein measured by Western blot analysis was not changed by raising plasma glucose and insulin in lean rats (1.00 ± 0.14 and 1.35 ± 0.19, respectively) or ZDF rats (1.04 ± 0.02 and 1.28 ± 0.18, respectively). The ratio of nuclear to cytoplasmic GKRP immunoreactivities was slightly (but not significantly) higher in ZDF rats (6.69 ± 1.31) than in lean rats (5.03 ± 0.43) and did not change with the rise in plasma glucose and insulin levels in either ZDF rats (6.56 ± 0.82) or lean rats (4.80 ± 0.64). The amount of GKRP protein was not changed in either lean rats (1.00 ± 0.10 and 0.94 ± 0.11, respectively) or ZDF rats (1.00 ± 0.11 and 1.10 ± 0.11, respectively).

Enzyme activities. Under the basal condition, HK and G-6-Pase activities were not different between ZDF and lean rats (Table 3). Total GK activity was higher in ZDF than in lean rats. The total activities and the percentage of the active form of glycogen synthase and phosphorylase were similar in ZDF and lean rats. The activities of HK, G-6-Pase, GK, total activity of glycogen synthase and phosphorylase, and percentage of the active form of glycogen phosphorylase seen with increased plasma glucose and insulin were similar to those seen under the

increase in ZDF rats. In ZDF rats, HGP, TGO, and GC did not change in response to elevated plasma glucose and insulin.

Glycogen content and glycogen synthesis in liver and skeletal muscle. Under basal conditions, glycogen content in the liver was significantly higher in ZDF than in lean rats (Fig. 3). The amounts of glycogen synthesized via the direct and indirect pathway at the end of experiments were similar in ZDF rats (1.58 ± 0.76 and 2.95 ± 1.12 µmol glucose/g liver, respectively) and in lean rats (0.95 ± 0.73 and 3.21 ± 2.02 µmol glucose/g liver, respectively). In response to the rise in plasma glucose and insulin, glucose incorporation via the direct pathway increased about fivefold in lean rats (4.97 ± 1.37 µmol glucose/g liver) but was unchanged in ZDF rats (1.41 ± 0.40 µmol glucose/g liver). Glycogen synthesis via the indirect pathway did not increase in either lean or ZDF rats (2.76 ± 1.00 and 3.07 ± 0.80 µmol glucose/g liver, respectively). In skeletal muscle, glycogen content and [³H]glucose incorporation into glycogen were similar in ZDF and lean rats.
basal condition in both lean and ZDF rats. The percentage of the active form of glycogen synthase seen increased when plasma glucose and insulin were higher in lean rats but was similar in ZDF rats compared with that under the basal condition.

**DISCUSSION**

The present study demonstrates an impairment of GK translocation from the nucleus to the cytoplasm in the liver of ZDF rats. Some in vivo studies (25, 62) have demonstrated that both GK and GKRP locate in higher density in the nucleus than in the cytoplasm in the presence of euglycemia and euinsulinemia. It has been demonstrated in normal rats that, in response to elevation of plasma glucose and/or insulin level within the physiological range (15) or refeeding (25), GK immunofluorescence markedly decreased in the nucleus and increased in the cytoplasm, indicating the translocation of GK from the nucleus to the cytoplasm. In support of these results, the present study demonstrates in lean rats that GK and GKRP are predominantly localized in the nucleus in the presence of euglycemia and euinsulinemia and that, following increased plasma glucose and insulin, GK immunofluorescence markedly decreased in the nucleus and increased in the cytoplasm. Because the total activity and protein content of GK measured in the liver homogenate were similar in the basal and the clamp conditions, the reduction of GK immunofluorescence in the nucleus seen in the clamp group was due to the export of GK into the cytoplasm rather than to an altered amount of GK. ZDF rats had a similar content of hepatic GK protein to that in normal rats. Regardless of the presence of hyperinsulinemia, however, GK was predominantly localized in the nucleus in the presence of euglycemia and euinsulinemia and that, following increased plasma glucose and insulin, GK immunofluorescence markedly decreased in the nucleus and increased in the cytoplasm. It is likely, therefore, that GK translocation from the nucleus to the cytoplasm in response to increased plasma glucose and insulin is impaired in ZDF rats.

GKRP seems to play a critical role in importing GK into the nucleus and in localizing GK in the nuclear compartment. Mice with engineered mutant null for GKRP exhibit cytoplasmic localization of GK even at low blood glucose levels (23, 32). When HeLa cells (18, 56) or human embryonic kidney cells (12) are transfected with the GK gene alone, cytoplasmic GK does not accumulate in the nucleus even in the presence of leptomycin B (56), which inhibits signal-mediated nuclear export (27, 39, 40). In contrast, when GK is coexpressed with GKRP, GK accumulates in the nucleus with GKRP (12, 56). These findings suggest that GKRP is essential for importing GK into the nucleus. Furthermore, an engineered mutant form of GK with reduced affinity for GKRP does not concentrate in
The nucleus even in the presence of GKR at low glucose levels (18, 56), suggesting that binding by GKR is essential for exporting GK to the cytoplasm. On the other hand, it is possible that GK translocation in response to increased plasma glucose and insulin, occurs by stimulating its dissociation from GKR.

Studies using cultured hepatocytes demonstrated that increased glucose concentration in the culture medium induced GK translocation from the nucleus to the cytoplasm (2, 13, 60, 62). Less is known about the molecular mechanism by which the decrease in extracellular glucose concentration causes translocation of GK from the nucleus. However, Agius and Stubbs (5) have demonstrated that a glucose analog that is not phosphorylated by HK family enzymes could cause GK translocation, suggesting that the mechanism does not involve metabolites of glucose or GK-catalyzed phosphorylation. If so, the effect of glucose may be mediated by increased intracellular concentration per se. The intracellular concentration of glucose in hepatocytes is equal to, or slightly higher than, the plasma concentration (64) and is rapidly equilibrated with a change in plasma glucose concentration in normal rats (47) because of the presence of a large number of GLUT2 transporters. Because the amount of GLUT2 in the liver of diabetic animals has been reported to be similar or higher compared with normal animals (51, 59), the

Table 3. Activities of HK, GK, and G-6-Pase and total activities and percentage of active form of glycogen synthase and phosphorylase at end of experiment in basal and clamp conditions in 6-h-fasted lean and ZDF rats

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Lean Basal</th>
<th>Lean Clamp</th>
<th>ZDF Basal</th>
<th>ZDF Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK, nmol.min⁻¹.mg protein⁻¹</td>
<td>5.92±0.39</td>
<td>6.19±0.47</td>
<td>4.69±0.43</td>
<td>5.75±1.32</td>
</tr>
<tr>
<td>GK, nmol.min⁻¹.mg protein⁻¹</td>
<td>12.85±1.18</td>
<td>12.68±0.97</td>
<td>16.79±1.03</td>
<td>18.71±2.34</td>
</tr>
<tr>
<td>G-6-Pase, nmol/min ⁴mg protein⁻¹</td>
<td>117.8±2.4</td>
<td>143.8±10.1</td>
<td>132.9±11.5</td>
<td>134.7±18.0</td>
</tr>
<tr>
<td>Total glycogen synthase, nmol.min⁻¹.mg protein⁻¹</td>
<td>5.50±0.19</td>
<td>6.91±0.23</td>
<td>6.58±0.54</td>
<td>5.38±0.49</td>
</tr>
<tr>
<td>Glycogen synthase, %active form</td>
<td>3.7±0.5</td>
<td>8.9±1.6†</td>
<td>3.2±0.5</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td>Total glycogen phosphorylase, nmol.min⁻¹.mg protein⁻¹</td>
<td>140.5±13.2</td>
<td>105.2±12.6</td>
<td>149.7±12.6</td>
<td>140.2±12.1</td>
</tr>
<tr>
<td>Glycogen phosphorylase, %active form</td>
<td>67.1±2.2</td>
<td>53.0±8.1</td>
<td>56.1±11.4</td>
<td>59.2±10.6</td>
</tr>
</tbody>
</table>

Values are means ± SE for 5 rats in each group. HK, hexokinase; GK, glucokinase; G-6-Pase, glucose-6-phosphatase. †P < 0.05 for lean basal vs. lean clamp group. There were no significant differences between the lean basal and ZDF basal groups and between the ZDF basal and ZDF clamp groups.

Fig. 5. A: immunohistochemical intracellular distribution of glucokinase (GK) and GK-regulatory protein (GKR) in the liver at the end of experiment in basal and clamp conditions in 6-h-fasted lean and ZDF rats. a: Lean basal group; b: lean clamp group; c: ZDF basal group; d: ZDF clamp group. Immunofluorescent staining for GK is revealed as red in the image; that for GKR is revealed as blue. Pink nuclear emission signal signifies the coexistence of GK with GKR in the nucleus; green image indicates the nucleus. Details of the procedure are described in RESEARCH DESIGN AND METHODS. B and C: quantitative intracellular distribution of GK and GKR, respectively, in basal and clamp conditions in 6-h-fasted lean and ZDF rats. N/C, nuclear-to-cytoplasmic pixel density ratios. Values are means ± SE as the ratio of nuclear to cytoplasmic immunofluorescence activities of GK and GKR for 5 rats in each group. †Significantly different from corresponding value under basal condition in identical animal type (P < 0.05).
unresponsiveness of GK translocation to increased plasma glucose may not result from a defect in increase in intracellular glucose concentration. Agius (1) showed that ethanol and glycerol inhibited the effect of glucose on GK translocation in cultured rat hepatocytes and suggested that an increase in the cytosolic NADH/NAD\(^+\) redox state inhibits GK translocation. In the present study, there was no difference in the plasma glyceral concentration between ZDF and lean rats. It is not known whether the intracellular redox state in the liver increased in ZDF rats. The mechanism by which GK translocation is impaired in ZDF rats remains to be determined.

In lean rats, as shown elsewhere in normal animals and humans, HGP was completely suppressed in response to increased plasma glucose and insulin levels. The rate of glucose incorporation into glycogen via the direct pathway was increased together with a threefold increase in the contribution of plasma glucose to form G-6-P, suggesting increased hepatic glucose uptake. Elevated GC is one of the features of premature and early stage type 2 diabetes (20, 21), whereas endogenous glucose production is reported to be similar to that in nondiabetic subjects (21, 28). An impairment of suppression of HGP by glucose and/or insulin has been repeatedly reported in patients and animals with type 2 diabetes (44, 45). Recently, lower splanchnic glucose uptake in response to hyperglycemic hyperinsulinemia has also been reported in patients in the prediabetic stage of type 2 diabetes (9, 10). Mevorach et al. (42) reported the lack of increasing cycling and unchanged flux through G-6-Pase in the face of a doubling in circulating glucose concentration in subjects with type 2 diabetes. Consistent with these findings in human and rat studies, under basal conditions ZDF rats exhibited markedly higher TGO and GC compared with lean rats, although endogenous glucose production rates were normal. They also have defects in suppression of HGP and TGO, a lack of increased glycogen synthesis from glucose via the direct pathway, and defects in increase in GC and contribution of plasma glucose to form G-6-P in response to increased plasma glucose and insulin. These features of impaired regulation of hepatic glucose flux observed in ZDF rats closely resemble those in subjects with type 2 diabetes.

To gain insight into GK flux, we measured several parameters that relate to GK flux, although we did not measure hepatic GK flux (glucose phosphorylation rate) directly. In the face of an increase in plasma glucose concentration, the contribution of plasma glucose to form G-6-P, intracellular G-6-P content, and calculated GC was not increased in ZDF rats even though there was no change in the flux through G-6-Pase (TGO) and no increase in the incorporation of G-6-P into glycogen. One of the most likely explanations for this is the failure of an increased plasma glucose concentration to enhance the flux through GK. Recent studies using cultured hepatocytes have shown that G-6-P arising from the catalytic action of GK is much more effective in mediating the activation of glycogen synthase than the same metabolite produced by HK I (26, 50, 55). Agius et al. (4) have shown that the rate of glycogen synthesis from glucose seen in cultured hepatocytes is very sensitive to small increases in total GK activity, as reflected in a high control coefficient (6.6 with 10 mM glucose) that is defined as the fractional increase in glucose incorporation into glycogen produced as a result of a fractional increase in the total GK activity induced by GK gene transfection using adenovirus. They also showed that the total GK activity, which caused a half-maximal increase in glycogen synthesis from glucose, is within the physiological range of hepatic GK activity (4). Seoane et al. (54) showed that impaired glycogen synthesis from glucose seen in cultured hepatocytes isolated from ZDF rats was overcome by overexpressing GK using adenovirus. Therefore a lack of increasing glycogen synthesis from plasma glucose is also suggestive of a defect in increase in GK flux.

On the other hand, an alternative explanation is theoretically possible. G-6-P is produced via glycogenolysis and gluconeogenesis in addition to glucose phosphorylation by GK. Even if glucose phosphorylation is increased, the contribution of plasma glucose to form G-6-P may not be changed if the G-6-P production rate via glycogenolysis and/or gluconeogenesis is increased in parallel with increased glucose phosphorylation rate. In this scenario, the G-6-P disposal rate has to be increased simultaneously in parallel with increased G-6-P production rate under steady state of metabolic flux. G-6-P is mainly disposed of through G-6-Pase, glycogen synthesis, and glycolysis. Because G-6-Pase flux (TGO) and the incorporation of glucose into glycogen were not increased, the increased G-6-P disposal should be due to increased glycolytic flux. Glycolytic flux is normally activated by the rise in glucose via increasing intracellular G-6-P concentration (35) and/or fructose 2,6-phosphate (F-2,6-P) by activating the 6-phosphofructo-2-kinase domain of the bifunctional enzyme (48). However, Seoane et al. (54) reported that, in the physiological range of glucose concentration, the glucose-induced increase in F-2,6-P concentration and lactate production were much lower in hepatocytes isolated from 12-wk-old ZDF rats than from lean rats. Interestingly, they also showed that GK overexpression using adenovirus overcame this defect seen in the diabetic hepatocytes (54). Rossetti et al. (53) demonstrated that hyperglycemia caused a marked inhibition of HGP, mainly through the suppression of glycogenolysis and the increase in GK flux in conscious rats. In addition, this group (Barzilai et al. (7)) showed that glucose-induced inhibition of HGP was impaired by inhibiting GK with glucosamine in normal rats, indicating that intact GK activity was required for the normal suppression of HGP by hyperglycemia. Insulin has been reported to stimulate glycolysis via increasing F-2,6-P by activating 6-phosphofructo-2-kinase domain of the bifunctional enzyme and via activating pyruvate kinase in the liver of normal animals (6). In ZDF rats, however, the liver, as well as other tissues (22), is resistant to insulin action, as shown by a lack of the activation of glycogen synthase (Table 3), impaired activation of Akt/protein kinase B, and blunted suppression of HGP (45, 46) in response to the rise in plasma insulin. These pieces of indirect evidence imply that marked impairments in the suppression of HGP and in hepatic glucose uptake in response to increased plasma glucose and/or insulin levels seen in ZDF rats result, at least in part, from a defect in activation of GK mediated by the rise in plasma glucose and/or insulin.

Even in normal animals, the physiological significance of GK translocation and its relation to GK activity is unknown. The volumes of the nucleus and the cytoplasm in hepatocytes are markedly different [the nuclear volume is only 10% of cell volume (65)], and the immunoreactivity reflects relative density (concentration) but not the absolute amount of protein in each compartment. Therefore, it is very difficult to assess the absolute amount of GK (both free and bound to GKRP)
It is undeniable that the unresponsiveness of glucose across the nuclear membrane through the nuclear pore system. We have demonstrated in the present study that GK translocation in the liver in response to the hyperglycemia and hyperinsulinemia is impaired in ZDF rats. In subjects with type 2 diabetes and a rodent model of type 2 diabetes, fructose, a precursor of F-1-P that stimulates GK dissociation from GKRPs, has been demonstrated to reduce postprandial glycemia (68) and increase hepatic glucose uptake (33). Thus it is possible that the impairment of short-term activation of GK via dissociating this enzyme from GKRPs and via subsequent translocation from the nucleus to the cytoplasm is one of the causes of the defect in hepatic glucose uptake in subjects with type 2 diabetes. Further study is required to understand the mechanism underlying impaired GK translocation in the liver of ZDF rats.

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