Glucotoxicity targets hepatic glucokinase in Zucker diabetic fatty rats, a model of type 2 diabetes associated with obesity

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Glucokinase (GK) plays a critical role in glucose effectiveness in the liver. The glucose-induced suppression of NHGP and increase in GS are associated with an augmentation of flux through GK without change in glucose-6-phosphatase (G-6-Pase) flux (35). Under low glucose concentrations, the binding of GK regulatory protein (GKRP) to GK inhibits GK activity by decreasing the affinity of the enzyme for glucose (46) and sequesters GK in the nucleus (2, 10, 15). An increase in glucose concentration dissociates GK from GKRP in a competitive manner and accelerates the translocation of GK from the nucleus to the cytoplasm, where glucose phosphorylation is catalyzed (2, 10, 15). This provides a mechanism for the acute effectiveness of glucose in regulating hepatic glucose flux. Insulin accelerates the glucose effect (3, 10, 45) and in addition regulates GK protein levels at the transcriptional level (23).

In studies of patients with T2DM, defective splanchnic glucose uptake and hepatic GS in response to hyperglycemia (4, 5) were associated with defective glucose phosphorylation (4, 27). The addition of a catalytic amount of fructose, a precursor of fructose-1-phosphate (F-1-P) that accelerates the dissociation of GK from GKRP, improved glucose intolerance (28) and nearly normalized the ability of hyperglycemia per se to suppress NHGP (21). Furthermore, some obese patients with T2DM have a marked decrease in the activity of GK compared with that in healthy subjects (8, 11). The catalytic properties of human GK and its inhibition by human GKRP are similar to that of rat GK (7). In Zucker diabetic fatty (ZDF) rats, a widely used genetic model of obese T2DM (39), defective suppression of NHGP and failure to increase flux from glucose to glycogen in response to a rise in plasma glucose and insulin are associated with defective augmentation of glucose phosphorylation (GK flux) resulting from impaired dissociation of GK from GKRP and subsequent decreased translocation of the enzyme from the nucleus to the cytoplasm in the early diabetic stage (15, 16, 36), abnormal relocation of GKRP to the cytoplasm in the presence of hyperglycemia during the middle stage (44), and a progressive reduction of hepatic GK protein levels in the

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**TYPE 2 DIABETES MELLITUS (T2DM)** is a disease characterized by persistent and progressive deterioration of glucose tolerance that is associated with loss of glucose effectiveness and development of insulin resistance (21, 27). The effectiveness of glucose refers to the ability of glucose per se to function as a negative feedback regulator in determination of blood glucose levels (6). In nondiabetic subjects, an acute rise in plasma glucose increases insulin secretion from pancreatic β-cells and via a mass action of glucose per se exerts an inhibitory effect on glucose production while stimulating glucose uptake (6, 42). On the other hand, patients with established T2DM fail to respond normally to elevated plasma glucose and/or insulin, resulting in impaired glucose uptake in peripheral tissues and a failure to suppress net hepatic glucose production (NHGP), both of which contribute to fasting hyperglycemia (13, 27). In addition, a defect in splanchnic glucose uptake accompanied by reduced hepatic glycogen synthesis (GS) contributes to excessive postprandial hyperglycemia (4, 5, 26). With a progressive loss of the ability of insulin and glucose to effectively regulate hepatic glucose flux, glycemic control deteriorates and the severity of diabetes increases.

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later stage of the disease (43). Therefore, defective glucose effectiveness on hepatic glucose flux in T2DM is likely associated with impaired GK activity. So far, the mechanism for the progressive deterioration of GK activity has not been clarified.

The contribution of persistent hyperglycemia to the deteriorating changes in hepatic glucose metabolism, referred to as glucotoxicity, has been implicated in patients with T2DM (21) as well as some diabetic animal models (16, 19, 24, 30), with normalization of hyperglycemia by treatment with insulin (21), phloridzin (24), or phlorizin analogs (16, 19, 30). Here, we show in ZDF rats that the correction of hyperglycemia, by initiating treatment with the renal sodium-glucose transporter 2 inhibitor (SGLT2-I) canagliflozin (31) in the midstage of diabetes, restored the glucose-induced augmentation of glucose phosphorylation as a result of the maintenance of GK protein levels with a normalized regulation of GK by GKRP. This in turn improved glucose effectiveness in regard to the suppression of NHGP and stimulation of GS. Our data provide a mechanism by which glucotoxicity reduces the effectiveness of glucose in regulating hepatic glucose flux in T2DM.

RESEARCH DESIGN AND METHODS

Animals. Six-week-old male ZDF rats (ZDF-Gmi-Crl-+/fa) and their lean littermates (ZCL; ZDF/GmiCrl-+/fa) were purchased from Charles River Laboratories (Wilmington, MA). Rats were fed with Formulab Diet 5008 (Purina Lab Diet; Purina Mills, Richmond, IN) and given water ad libitum in an environmentally controlled room with a 12:12-h light-dark cycle. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of both the US Department of Agriculture and the National Institutes of Health, with all protocols receiving approval from the Vanderbilt University Institutional Animal Care and Use Committee.

Measurement of change in protein and mRNA levels of GK and phosphoenolpyruvate carboxykinase in liver during the progression of diabetes. Blood and tissue samples were collected from ZDF and ZCL rats fasted for 6 h from 7 AM to 10–11, 14–15, 20–22, and 26–28 wk of age (Fig. 1A). Correction of hyperglycemia in ZDF rats. ZDF rats, which had >12 mM blood glucose levels after a 6-h fast at 14 wk of age, were treated with either vehicle (0.2% carboxymethyl cellulose containing 0.2% Tween-80) at 5 mg/kg [ZDF rats diabetic fatty rats treated with vehicle (ZDF-V)] or the SGLT2-I canagliflozin (Mitsubishi Tanabe Pharma, Saitama, Japan) (31) at 10 mg/kg [ZDF rats treated with canagliflozin (ZDF-CA)] by gavage at 4 PM daily from 14 wk of age.

Measurement of fasting metabolic profile. Just prior to (14 wk old) and at 1 (15 wk old), 4 (18 wk old), and 6 wk (20 wk old) of the treatment with either vehicle or SGLT2-I, the animals were fasted from 6 AM to 6 h, and blood was collected from the tail vein (Fig. 1B). To perform indirect calorimetry and body composition analyses, rats were adapted to the system for 3 days before each measurement prior to and after 1, 4, and 6 wk of treatment with either vehicle (5 ml/kg·day−1) or SGLT2-I (10 mg/kg·day−1) (Fig. 1C). Food intake, oxygen consumption, and carbon dioxide production were monitored for 1-min intervals every 12 min for 24 h using Oxymax-CLAMS (Columbus Instruments, Columbus, OH). Body composition was measured using EchoMRI 700 (Echo Medical Systems, Houston, TX). To collect urine at 5 wk of the treatment, rats were housed in metabolic cages (Harvard Apparatus, Holliston, MA) for 3 days, and urine was collected during the last 24 h.

Measurement of postprandial metabolic profiles. Two weeks before each study (Fig. 1, C and D), surgery was performed to place sterile silicon rubber catheters in the left common carotid artery, the right external jugular vein, and the stomach, as described previously (10, 15, 16, 38). Mixed-meal tolerance (MTT) studies were conducted in 20-h-fasted conscious rats that were treated with either vehicle (5 ml/kg·day−1) or SGLT2-I (10 mg/kg·day−1) from 14 wk of age for 6 wk (Fig. 1E). Each MTT experiment (Fig. 1F) consisted of a 30-min control period (−30 to 0 min) and a 180-min test period (0−180 min). At 0 min, a liquid mixed meal (6.23 g of glucose, 2.99 g of protein, and 0.84 g/20 ml containing [3-3H]glucose (50 μCi) and [U-14C]glucose (50 μCi) was infused into the stomach through the gastric catheter at 400 ml/kg·min−1 for 30 min after an 8-ml/kg bolus. Blood samples were collected from the arterial catheter. Urine was collected during the test period and from the bladder at the end of the test period.

Hyperinsulinemic hyperglycemic clamp studies. The clamp studies (Fig. 1G) were conducted in 6-h-fasted conscious rats that were treated with either vehicle (5 ml/kg·day−1) or SGLT2-I (10 mg/kg·day−1) from 14 wk of age for 1 (Fig. 1D) or 6 wk (Fig. 1E). These rats had their last dosing 45 h before each study. The surgery to implant the catheters was performed 2 wk before each clamp study. Each clamp study consisted of a 90-min tracer equilibration period (−150 to −60 min), a 60-min basal period (−60 to 0 min), and a 120-min test period (0−120 min). At −150 min, both [2-14H] - and [3-3H]glucose were given at 60 μCi as a bolus through the jugular vein catheter, followed by continuous infusion at 0.6 μCi/min. During the test period, somatostatin was infused through the jugular vein catheter at 5 μg·kg−1·min−1 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 μU·kg−1·min−1 and 2.6 ng·kg−1·min−1, respectively. Plasma glucose levels were kept at <22 mM by infusing a 50% glucose solution into the systemic circulation through the jugular vein catheter at a variable rate. At 120 min, [U-14C]alanine was given at 100 μCi in a bolus through the arterial catheter, followed by continuous infusion at 10 μCi/min. Blood samples were taken from the arterial catheter. Blood glucose levels were monitored using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN). At each sampling time, collected erythrocytes were resuspended in saline and given back to the test animal during the study.

Tissue collection from animals. At the end of each study, rats were anesthetized with arterial infusion of pentobarbital sodium (60 mg/kg), and a laparotomy was performed immediately. The median lobe of the liver was excised and dropped into ice-cold PBS containing 4% paraformaldehyde for immunohistological analysis. The left lobe of liver and skeletal muscle (vastus lateralis, gastrocnemius-plantalis, and soleus) were frozen using Wollenberger tongs precooled in liquid nitrogen.

Measurements of protein and mRNA levels of GK, GKRP, and phosphoenolpyruvate carboxykinase. GK, GKRP, and phosphoenolpyruvate carboxykinase (PEPCK) protein levels in liver were measured using Western blot analysis, as reported previously (15, 16). RNA was extracted using a Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories). The abundance of transcripts was assessed by real-time PCR, using a SYBR Green Supermix (Bio-Rad Laboratories) with sense and antisense primers, as listed in Table 1. Quantities of specific mRNAs of interest were normalized to that of ribosomal protein 13a for each gene.

GK and GKRP immunostaining. Measurements of the immunoreactivity of both GK and GKRP in the nucleus and the cytoplasm in each parenchymal cell were performed as described previously (10, 15, 16, 36).

Enzyme activities in liver and metabolites in blood and tissues. We measured glycogen synthase activity and phosphorylase activity, glycogen and glucose 6-phosphate (G-6-P) in liver, plasma levels of glucose, free fatty acids (FFAs), triglyceride, insulin, and glucagon, corticosterone, blood levels of hemoglobin A1c, lactate, and alanine, urinary glucose, specific activities of [2-14H]glucose, [3-3H]glucose, and [14C]glucose in plasma glucose and glycogen-glucose, the liver content of uridine 5′-diphosphate glucose (UDPG) and phosphoenol-
pyruvate (PEP), and the radioactivity of [3H] and [14C] in each fraction, as described previously (15, 16, 44). Triglyceride content in liver, soleus muscle, and gastrocnemius muscle were determined according to the method of Carr et al. (9).

Calculations. Rates of glucose appearance (Ra) and disappearance (Rd), endogenous glucose production (EGP), glucose cycling (GC), G-6-Pase flux, GS in liver and skeletal muscle, and percent contribution of plasma glucose and PEP to form UDPG in liver were calculated as described previously (44).

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 evaluated using an unpaired Student t-test. Probabilities <5% (P < 0.05) were considered to be statistically significant.

RESULTS

Change in protein and mRNA levels of GK and PEPCK in liver during the progression of diabetes. At 10–11 wk of age, GK protein levels were similar despite markedly higher GK...
mRNA levels in ZDF compared with ZCL rats. As diabetes progressed in the ZDF rats, plasma insulin levels fell (Fig. 2A) while fasting hyperglycemia developed (Fig. 2B), and both GK mRNA (Fig. 2C) and protein (Fig. 2D) levels decreased. However, although GK protein levels decreased to 30% of the levels in ZDF rats by 26–28 wk of age, GK mRNA levels were still maintained at the levels seen in ZCL rats. In contrast to GK, GKRP protein remained unchanged in all groups (Fig. 2F), whereas GKRP mRNA tended to be lower in ZDF compared with ZCL rats (Fig. 2E) during the development of diabetes. PEPCK is an enzyme that is generally regulated opposite to that of GK (20). PEPCK protein levels increased with age and severity of diabetes (Fig. 2H), whereas PEPCK mRNA remained stable (Fig. 2G).

Effect of treatment with SGLT2-I on fasting and postprandial metabolism in ZDF rats. Compared with ZCL rats, at 14 wk of age ZDF rats had nearly twice the daily food intake (Fig. 3A), ~10% greater body weight (Fig. 3B), a higher percent body fat (Fig. 3, E and G), lower lean mass (Fig. 3, F and H), and similar daily oxygen consumption (Fig. 3C) with lower respiratory quotient (Fig. 3D). During the following 6 wk, body weight gain was lower in ZDF-V than in ZCL rats treated with vehicle (ZCL-V) but was restored in ZDF-CA rats with increased body fat mass (Fig. 3, E and G). On the other hand, treatment with SGLT2-I did not alter any other parameters. Rates of daily urination (Fig. 3I) and urinary glucose excretion (Fig. 3J) were markedly higher in ZDF-V and ZDF-CA com-

![Fig. 2. Alterations in glucokinase (GK), glucokinase regulatory protein (GKRP), and phosphoenolpyruvate carboxykinase (PEPCK) mRNA and protein levels in liver throughout the development of diabetes in Zucker diabetic fatty (ZDF) rats. Animals were fasted for 6 h from 7 AM before collection of blood and tissue. Changes with age and severity of diabetes are reported for plasma insulin (A) and glucose (B) and levels of GK mRNA (C) and protein (D), GKRP mRNA (E) and protein (F), and PEPCK mRNA (G) and protein (H). RNA and protein levels in ZDF rats (black bars) are expressed relative to those measured in age-matched lean littermates of ZDF (ZCL) rats (open bars). Values are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of the ZCL group (P < 0.05).](http://ajpendo.physiology.org/Content/Full/doi:10.1152/ajpendo.00507.2013)
levels tended to rise progressively to 25.3 in ZDF-V rats, plasma glucose and markedly higher Hb A1c levels after 6 wk (20 wk of age), and after initiation of treatment with vehicle or SGLT2 inhibitor for 1 (15 wk of age), 4 (18 wk of age), and 6 wk (20 wk of age) of treatment. Body weight and body composition, reported as %fat mass (E), %lean mass (F), fat weight (G), and lean weight (H), were measured just prior to and after 1, 4, and 6 wk of either vehicle or SGLT2 inhibitor treatment. Data are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of the ZCL group (P < 0.05); †significant difference from the values at week 0 in the identical group (P < 0.05).

To examine the role of chronic hyperglycemia in the progressive reduction of GK protein and mRNA levels, as well as the abnormal intracellular localization of GK protein in the livers of ZDF rats, hyperglycemia was chronically corrected by treatment with SGLT2-I. Under short-term fasting conditions, ZDF rats at 14 wk of age (just before the initiation of SGLT2-I treatment), compared with age-matched ZCL rats, exhibited marked fasting hyperglycemia (22.6 ± 1.9 vs. 7.1 ± 0.4 mM; Fig. 4A) despite the presence of nearly three times higher plasma insulin levels (Fig. 4C) and similar plasma glucagon levels (Fig. 4D). In ZDF-V rats, plasma glucose levels tended to rise progressively to 25.3 ± 0.8 mM (Fig. 4A), along with a progressive decrease in plasma insulin level (Fig. 4C) and markedly higher Hb A1c levels after 6 wk (20 wk of age) of the treatment, compared with ZCL-V rats (Fig. 4B). Treatment with SGLT2-I (ZDF-CA) corrected fasting hyperglycemia within 1 wk, maintained plasma glucose levels at nearly normal levels (Fig. 4A), and lowered Hb A1c levels (Fig. 4B) without affecting plasma levels of insulin (Fig. 4C), glucagon (Fig. 4D), corticosterone (Fig. 4E), FFAs (Fig. 4G), triglycerides (Fig. 4H), glycogen content in the vastus lateralis muscle (Fig. 4J), or triglyceride content in liver (Fig. 4K) and the soleus muscle (Fig. 4L). Treatment with SGLT2-I partially restored blood lactate levels (Fig. 4F) and hepatic glycogen content (Fig. 4I) after 1 and 6 wk and completely restored triglyceride content in gastrocnemius-plantaris muscle (Fig. 4L) after 6 wk of the treatment.

At 14 wk of age, compared with ZCL rats, GK protein in liver of ZDF rats was ~80% of that in ZCL rats, albeit not significantly different between the groups. GK mRNA levels tended to be higher. After 6 wk, at 20 wk of age, GK protein in ZDF-V rats (Fig. 5B) decreased by 60% (Fig. 5, B and I), whereas GK mRNA remained stable (Fig. 5A). In ZDF-CA, GK protein levels were maintained (Fig. 5, B and I) without
GK mRNA levels being altered (Fig. 5A). In contrast to GK, GKRP protein remained unchanged in all groups (Fig. 5D), whereas GKRP mRNA tended to be lower in ZDF-V and ZDF-CA compared with ZCL-V rats (Fig. 5C) during the development of diabetes. The PEPCK protein levels with respect to mRNA levels were higher in ZDF-V compared with ZCL-V rats, but neither parameter differed in the liver (Fig. 5K) and vastus-lateralis (V-L) muscle (Fig. 5L) of either vehicle or SGLT2 inhibitor treatment. Data are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of the ZCL group (P < 0.05); †significant difference from the corresponding values of the ZDF-V group (P < 0.05); ‡significant difference from the values at week 6 in the identical group (P < 0.05).
by correcting hyperglycemia was likely not the result of altered insulin action leading to greater gene transcription but possibly due to an effect on protein synthesis and/or degradation.

Effect of correction of hyperglycemia on glucose flux and GK activity. We also examined whether glucose-induced dissociation of GK from GKRP and subsequent translocation of GK from the nucleus to the cytoplasm is restored by correcting hyperglycemia, which could normalize localization of GK and GKRP and maintain GK protein levels. In ZCL-V rats, during the hyperglycemic hyperinsulinemic clamp after 1 (Fig. 7) and 6 wk (Fig. 8) of vehicle treatment, compared with that during nonclamp basal conditions (Fig. 5, E, F, and J), the nuclear localization of GK decreased markedly, as indicated by a N/C drop from ~2 to 1 during the clamp after both treatment lengths, whereas GKRP localization remained primarily nuclear (Fig. 7, J, M, and N, and Fig. 8, J, M, and N, compared with Fig. 5, E, F, and J). In ZDF-V rats, the GK and GKRP immunoreactivity found within the nucleus relative to that in the cytoplasm (N/C) was markedly lower under nonclamp basal conditions (Fig. 5, E, F, and J) and did not change during hyperglycemic hyperinsulinemic clamp (Figs. 7, J, M, and N, and 8, J, M, and N). In ZDF-CA rats, similar to ZCL-V rats, the N/C of GK decreased markedly during the clamp after both treatment lengths, whereas GKRP localization remained primarily nuclear (Figs. 7, J, M, and N, and 8, J, M, and N). To avoid the confusion, the sentences have been improved. These results

Fig. 5. Hepatic expression of GK, GKRP, and PEPCK as well as intracellular localization of GK and GKRP in ZDF rats before and after 6 wk of treatment with either vehicle or SGLT2 inhibitor. ZCL and ZDF rats were treated with vehicle [ZCL-V (open bars) and ZDF-V (gray bars)] or an SGLT2 inhibitor (ZDF-CA; black bars) once every day from 14 wk of age for 6 wk.

A. GK mRNA
B. GK Protein
C. GKRP mRNA
D. GKRP Protein
E. GK
F. GKRP
G. PEPCK mRNA
H. PEPCK Protein
I. Western blot
J. Intracellular localization of GK and GKRP

Values are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of the ZCL group (P < 0.05); †significant difference from the corresponding values of the ZDF-V group (P < 0.05); significant difference from the values at just before the treatment (14 wk of age) in the identical group (P < 0.05).
indicate that correction of hyperglycemia nearly normalized nuclear localization of GK and GKRP under basal conditions and restored the dissociation of the GK-GKRP complex, followed by GK translocation from the nucleus to the cytoplasm in response to hyperglycemic hyperinsulinemia in ZDF rats.

To assess whether the restoration of glucose-induced dissociation of the GK-GKRP complex followed by GK translocation from the nucleus to the cytoplasm in ZDF-CA rats is accompanied by activation of GK, the relationship between hepatic glucose phosphorylation and intermediate flux was analyzed. Under basal conditions after 1 and 6 wk of treatment (Figs. 7 and 8 and Table 3), in ZCL-V rats, EGP was completely suppressed within 30 min and substantial GS occurred, accompanied by an approximately fourfold increase in GC and a 74% contribution of plasma glucose to form UDPG. In ZDF-V rats, the glucose infusion rate required to maintain hyperglycemia was markedly lower compared with ZCL-V rats. EGP decreased by only ~40%, and total GS was only ~23% of that seen in ZCL-V (Table 3). These defects were accompanied by a defective increase in GC (Fig. 7F) as well as a decrease in G-6-P content and in percent contribution of plasma glucose to form UDPG, G-6-P, and glycogen (Table 3). These indicate decreased glucose phosphorylation compared with that of ZCL-V rats. G-6-Pase fluxes were not significantly different from those in ZCL rats, and there was less activation of glycogen synthase. In ZDF-CA compared with ZDF-V rats, EGP was completely suppressed with increased percent contribution of GC and decreased percent

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**Fig. 6. Effect of SGLT2 inhibitor on metabolic responses to a gavage of a liquid mixed meal.** ZCL and ZDF rats were treated with vehicle [ZCL-V (● and open bars) and ZDF-V (● and gray bars)] or an SGLT2 inhibitor (ZDF-CA; ● and black bars) once every day from 14 wk of age for 6 wk. The last treatment was given 2 days before each study, and animals were fasted for 20 h before the MTT. Plasma levels of glucose (A), insulin (B), glucagon (C), and FFAs (D), as well as blood lactate levels (E), were measured for 30 min and just prior to a gavage-administrated mixed meal from 0 to 30 min and then at the indicated times ±3 h during and after the meal. Urinary glucose excretion (F) was measured during the 3-h postmeal study period. Glycogen synthesis in liver (G) and muscle (H) as well as liver activities of glycogen synthase (I) and glycogen phosphorylase (a (J) were measured at the end of the 3-h MTT. The relative abundance of GK mRNA in ZCL-V, ZDF-V, and ZDF-CA rats after MTT is illustrated (K). Values are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of the ZCL group (P < 0.05); †significant difference from the corresponding values of the ZDF-V group (P < 0.05).
Fig. 7. Metabolic parameters prior to and during hyperglycemic hyperinsulinemic clamp of ZCL and ZDF rats treated with vehicle [ZCL-V (● and open bars) and ZDF-V (● and gray bars)] or an SGLT2 inhibitor (ZDF-CA; ● and black bars) once every day from 14 wk of age for 1 wk. Time course of plasma glucose (A), plasma insulin (B), plasma glucagon (C) rates for glucose infusion (D), endogenous glucose production (E), glucose cycling (F), glucose disappearance (G), blood lactate (H), and plasma FFAs (I) during a hyperglycemic hyperinsulinemic clamp in ZCL-V, ZDF-V, and ZDF-CA are shown. J: images from livers collected from the animals at the end of the clamp study protocol depict fluorescence due to GK immunoreactivity (red), GKRP immunoreactivity (blue), and DNA (green). The ratios of immunoreactivity in the nucleus to that in the cytoplasm of GK (M) and of GKRP (N) are reported after image analysis of fluorescently stained liver slices (J). GK (K) and GKRP (L) protein levels are expressed relative to those measured in ZCL rats. Values are means ± SE of 6 animals in each group. *Significant difference from the corresponding values of ZCL-V group (P < 0.05). †Significant difference from the corresponding values of the ZDF-V group (P < 0.05); ‡significant difference from the values at basal in the identical group (P < 0.05).
contribution of gluconeogenesis to G-6-Pase flux, whereas total flux through G-6-Pase was not changed. GS was partially restored (Fig. 7 and Table 3), and the restoration was due to increased incorporation of plasma glucose into glycogen via the direct pathway. These improvements were accompanied by partial restoration of G-6-P content and the percent contribution of plasma glucose to form UDPG. Endogenous glucose disposal rate could theoretically be estimated as the difference
between $R_d$ and glucose excretion rate into urine. In this study, since glucose excretion into urine was measured as cumulated amount of glucose in urine collected during the clamp period, glucose excretion rate into urine at each time point could not be measured. In ZDF-V rats, the increase in $R_d$ in response to hyperinsulinemia-hyperglycemia after 1 and 6 wk of treatment was markedly smaller compared with ZCL-V rats (Figs. 7 and 8 and Table 3). Cumulated glucose excretion into urine was similar and smaller relative to $R_d$ in both groups; therefore, in this situation, endogenous glucose disposal rate might be substantially lower in ZDF-V compared with ZCL-V rats. In ZDF-CA compared with ZCL-V rats, whereas the increase in $R_d$ was completely restored, the cumulated glucose excretion into urine was markedly higher, implying that endogenous glucose disposal rate likely remained low. Since both $R_d$ and cumulated glucose excretion into urine were markedly higher compared with ZDF-V rats, it is not clear whether the endogenous glucose disposal rate was improved by SGLT2-I treatment.

**DISCUSSION**

We demonstrated that correction of persistent hyperglycemia from the middle stage of diabetes normalized all three different types of impairments of GK activity that occur in the liver of ZDF rats during progression of the disease. These are a defect of glucose-induced GK translocation from the nucleus to the cytoplasm, abnormal levels of intracellular residency of GKRPs in the cytoplasm, and an eventual reduction of GK protein level. Reducing hyperglycemia corrected the defect in the suppression of NHGP and the rate of GS in response to hyperglycemic hyperinsulinemia. This was associated with restored augmentation of glucose phosphorylation, which is likely the result of maintaining GK protein expression and the normalization of GK translocation in response to hyperglycemia. These results suggest that glucotoxicity alters GK regulation and expression, which leads to an inability of liver to detect elevated plasma glucose and thereby a loss in the effectiveness of glucose to regulate liver glucose flux in T2DM.

**Glucotoxicity impairs an allosteric regulation of GK by GKRPs and reduces GK protein in liver.** Glucose induces GK translocation by causing the dissociation of GK from GKRPs by the binding of glucose to the catalytic site of GK (2, 46). Previously, we reported in ZDF rats at an early stage of diabetes that an intraperitoneal infusion of a small amount of sorbitol, which is a precursor of F-1-P, a ligand of GKRPs that reduces GKRPs binding affinity for GK (46), could initiate translocation of GK from the nucleus to the cytoplasm (36). This suggested that defective GK translocation in response to hyperglycemic hyperinsulinemia in ZDF rats at this early stage of diabetes is likely due to impaired glucose-induced dissociation of the GK-GKRPs complex. In contrast, at the middle stage of diabetes in the ZDF rats used in our current study, GK is not sequestered in the nuclear compartment by GKRPs but is distributed evenly between the nuclear and cytoplasmic compartments. Additionally, GKRPs intracellular localization was reduced in the nuclear and increased in the cytoplasmic compartment. As a result of these abnormal basal intracellular distributions of GK and GKRPs, there was a failure to detect a translocation of GK in response to hyperglycemic hyperinsulinemia.

Altered distribution of GK and GKRPs was described previously in cultured cells (37), wherein GKRPs was homogeneously distributed between the cytoplasmic and nuclear compartments.
Table 3. Glucose flux in liver and skeletal muscle, plasma hormones, and metabolites as well as hepatic expression and intracellular distribution of GK and GKRP in ZCL and ZDF rats, as measured during the test period of the clamp studies after 1 (15 wk of age) and 6 wk (20 wk of age) of daily treatment with vehicle or an SGLT2 inhibitor

<table>
<thead>
<tr>
<th>Groups</th>
<th>After 1 Wk of Treatment</th>
<th>After 6 Wk of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZCL-V</td>
<td>ZDF-V</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>21.9 ± 0.2</td>
<td>22.9 ± 1.1</td>
</tr>
<tr>
<td>Plasma insuln, ng/ml</td>
<td>3.91 ± 0.61</td>
<td>3.90 ± 0.44</td>
</tr>
<tr>
<td>Plasma glucagon, pg/ml</td>
<td>43.8 ± 1.2</td>
<td>45.8 ± 2.2</td>
</tr>
<tr>
<td>Blood lactate, mM</td>
<td>1.72 ± 0.28</td>
<td>1.90 ± 0.25</td>
</tr>
<tr>
<td>Plasma FFAs, mM</td>
<td>0.13 ± 0.01</td>
<td>0.31 ± 0.08*</td>
</tr>
<tr>
<td>Glucose infusion rate, μmol·kg⁻¹·min⁻¹</td>
<td>203 ± 10</td>
<td>39 ± 10*</td>
</tr>
<tr>
<td>Glucose excretion into urine, mmol·kg⁻¹·130 min⁻¹</td>
<td>3.27 ± 0.46</td>
<td>3.26 ± 0.20</td>
</tr>
<tr>
<td>EGP, μmol·kg⁻¹·min⁻¹</td>
<td>−9.7 ± 0.97</td>
<td>36.0 ± 8.8*</td>
</tr>
<tr>
<td>Ra, μmol·kg⁻¹·min⁻¹</td>
<td>187 ± 9</td>
<td>75 ± 10*</td>
</tr>
<tr>
<td>Glucose-6-phosphatase flux, μmol·kg⁻¹·min⁻¹</td>
<td>Total</td>
<td>94.2 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>From plasma glucose</td>
<td>73.8 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>From PEP</td>
<td>25.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>From others</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Contribution to form UDP glucose, %</td>
<td>Total</td>
<td>137 ± 13</td>
</tr>
<tr>
<td></td>
<td>From plasma glucose</td>
<td>100 ± 8</td>
</tr>
<tr>
<td></td>
<td>From PEP</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>Intermediates in liver</td>
<td>Glycogen, μmol glucose/g</td>
<td>207 ± 11</td>
</tr>
<tr>
<td></td>
<td>UDP glucose, μmol/g</td>
<td>217 ± 13</td>
</tr>
<tr>
<td></td>
<td>Glucose 6-phosphate, μmol/g</td>
<td>159 ± 15</td>
</tr>
<tr>
<td></td>
<td>PEP, μmol/g</td>
<td>56.0 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>GS in skeletal muscle from plasma glucose, μmol/g</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>Muscle glycogen, μmol glucose/g</td>
<td>30.2 ± 2.6</td>
<td>28.5 ± 2.7</td>
</tr>
<tr>
<td>Glycogen synthase in liver</td>
<td>Total, U/g</td>
<td>2.32 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Active form, U/g</td>
<td>0.70 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>Active/to total</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>Glycogen phosphorylase (active form) in muscle, U/g</td>
<td>23.4 ± 2.6</td>
<td>27.4 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 clamp experiments for each group. UDP, uridine 5'-diphosphate; PEP, phosphoenolpyruvate. Animals were fasted for 6 h before each clamp study. The averages of the values measured at 75, 90, 105, 120, and 130 min during the test period of the clamp study are reported. *Significant difference from the corresponding values of the ZCL group (P < 0.05); †significant difference from the corresponding values of the ZDF group (P < 0.05).

parts when this protein was expressed in cells in the absence of GK. Coexpression of GK and GKRP caused predominant localization of both proteins in the nucleus, implying that normally the binding between GK and GKRP is necessary for predominant residency of both GK and GKRP in the nucleus (37). So far, it remains unknown whether GK forms a complex with GKRP or is dissociated from it when they both reside in the cytoplasm, as occurs during the middle stage of diabetes in ZDF rats.

Treatment of ZDF with SGLT2-I (ZDF-CA) prevented chronic hyperglycemia, which also resulted in restoring GK and GKRP to intracellular distributions similar to that of ZCL-V. In addition, GK translocation in response to hyperglycemic hyperinsulinemia was restored in ZDF-CA. This restoration of GK translocation by the correction of hyperglycemia was not accompanied by a simultaneous decrease in phosphorylase a activity, a primary metabolic effect of insulin on liver, suggesting no improvement of insulin resistance. Therefore, it is likely that glucotoxicity directly impairs intracellular residency of GKRP and glucose-induced GK translocation.

Insulin regulates GK protein levels at the level of gene transcription (23). GK mRNA levels in liver were decreased in line with the decrease in fasting insulin levels during the development of diabetes. GK protein levels were disproportionately reduced relative to its mRNA levels at all stages of diabetes compared with age-matched ZCL controls. Further, prevention of the progressive decrease in GK protein in liver by correcting hyperglycemia was not accompanied by an alteration of GK mRNA levels in either the fasting or postprandial states, which suggests that glucotoxicity decreases GK protein by affecting posttranscriptional processes. Studies using mice that do not express GKRP (GKRP gene ablated) (14, 18) have suggested that a lack of GKRP protein and/or its ability to sequester GK to the nuclear compartment will decrease GK protein levels without changes in GK mRNA levels in liver. In ZDF rats, the reduction of GK protein began around 12–13 wk of age, when the shift of residency of GKRP to the cytoplasm was initiated. Correcting hyperglycemia from 14 wk of age restored normal nuclear localization of GKRP within a week. Therefore, it is likely that, in ZDF rats, persistent localization of GK in the cytoplasm accelerates GK protein degradation and thus eventually results in and/or contributes to lower GK protein levels. The mechanism by which glucotoxicity impairs normal intracellular residency of GKRP, glucose-induced GK translocation, and maintenance of GK protein levels remains to be studied.
Glucotoxicity impairs hepatic glucose flux by affecting GK activity in ZDF rats. So far, there is no method for direct measurement of the glucose phosphorylation rate in vivo. Under steady-state conditions, if the liver exhibits an increased rate of glucose phosphorylation, the resulted increase in the flux of plasma glucose into the G-6-P pool would cause increased content of this metabolite that in turn would drive flux into downstream pathways (G-6-Pase flux, GS, and glycolysis). This increased flux should be accompanied by increases in the percent contribution of plasma glucose to form these metabolites in these downstream pathways. The defects in suppression of NHGP and stimulation of GS seen in response to hyperglycemic hyperinsulinemia in ZDF-V were accompanied by decreases in G-6-P content, partitioning of plasma glucose to form G-6-P, and flux of G-6-P derived from plasma glucose toward both glucose (GC) and glycogen. On the other hand, total G-6-Pase flux was not increased in ZDF-V compared with ZCL-V and not altered by correcting hyperglycemia in ZDF-CA. Therefore, the defects in suppression of NHGP and stimulation of GS seen in response to hyperglycemic hyperinsulinemia in ZDF-V were associated with decreased augmentation of GK flux. The properties and capacity of the liver’s GLUT2 are such that glucose transport across the plasma membrane depends on its concentration gradient, and therefore, it is not a regulatory step in determination of hepatic glucose flux (29, 41). The catalytic activity of GK is not affected by its product (G-6-P) (32), and indeed, GK flux is not altered by a change in the G-6-P level resulting from a change in flux of the downstream pathways (44). Therefore, the improvement of the liver’s ability to suppress glucose production and store glucose as glycogen in response to the rise in plasma glucose achieved by correcting hyperglycemia was due to normalized acceleration of glucose phosphorylation that might relate to maintenance of GK protein expression and normalization of GK translocation in response to hyperglycemia. Since the correction of hyperglycemia did not alter elevated plasma levels of FFAs, corticosterone, insulin, and glucagon, which have been reported to contribute to the lack of glucose effectiveness on liver (21, 22, 25, 42) and/or skeletal muscle (22) in T2DM, glucotoxicity appears to directly affect the liver.

Therapeutic benefit of SGLT2-I. Since hyperinsulinemia and insulin resistance precede and predict the subsequent development of T2DM (47), chronic hyperglycemia is unlikely to be the cause of the initial insulin resistance. However, once diabetes has developed, the chronic existence of hyperglycemia (glucotoxicity) may contribute to the vicious cycle, which worsens insulin resistance and glucose effectiveness, and may lead to diabetic complications (40). Therefore, breaking the vicious cycle by correcting hyperglycemia with a SGLT2-I is likely to be an attractive strategy to ameliorate diabetes (1, 17). On the other hand, since in insulin-resistant diabetic patients (48) and animals (12, 47) hyperglycemia compensates for insulin resistance and glucose intolerance to allow utilization and storage of absorbed glucose at nearly normal levels, one might be concerned that the SGLT2-I strategy, which normalizes hyperglycemia by discarding glucose into urine, might impair carbohydrate storage in such patients. Interestingly, contrary to such expectations, ZDF treated with SGLT2-I did not exhibit a symptom of glucose deficiency, such as increased lipid oxidation or decreased glycogen storage, because of the concomitant improvement in glucose tolerance due to the elimination of glucotoxicity.

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