Restoration of Hepatic Glucokinase Expression Corrects Hepatic Glucose Flux and Normalizes Plasma Glucose in Zucker Diabetic Fatty Rats

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OBJECTIVE—We examined in 20-week-old Zucker diabetic fatty (ZDF) rats whether restoration of hepatic glucokinase (GK) expression would alter hepatic glucose flux and improve hyperglycemia.

RESEARCH DESIGN AND METHODS—ZDF rats were treated at various doses with an adenovirus that directs the expression of rat liver GK (AdvCMV-GKL) dose dependently, and various metabolic parameters were compared with those of nondiabetic lean littermates (ZCL rats) before and during a hyperglycemic clamp. Viral infection per se did not affect hepatic GK activity, since expression of a catalytically inactive form of GK did not alter endogenous hepatic GK activity.

RESULTS—ZDF rats compared with ZCL rats have lower hepatic GK activity (11.6 ± 1.9 vs. 32.5 ± 3.2 mU/mg protein), marked hyperglycemia (23.9 ± 1.2 vs. 7.4 ± 0.3 mmol/l), higher endogenous glucose production (80 ± 3 vs. 38 ± 3 μmol·kg⁻¹·min⁻¹), increased glucose-6-phosphatase flux (150 ± 11 vs. 58 ± 8 μmol·kg⁻¹·min⁻¹), and during a hyperglycemic clamp, a failure to suppress endogenous glucose production (80 ± 7 vs. −7 ± 4 μmol·kg⁻¹·min⁻¹) and promote glucose incorporation into glycogen (15 ± 5 vs. 43 ± 3 μmol·g liver⁻¹).

TREATMENT of ZDF rats with different doses of AdvCMV-GKL, which restored hepatic GK activity to one to two times that of ZCL rats, normalized plasma glucose levels and endogenous glucose production. During a hyperglycemic clamp, glucose production was suppressed and glucose incorporation into glycogen was normal.

CONCLUSIONS—Alteration of hepatic GK activity in ZDF rats has profound effects on plasma glucose and hepatic glucose flux.

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In Western society, obesity is generally considered a primary risk for individuals with type 2 diabetes, since 60–90% of type 2 diabetic subjects are also obese (1). Initially, obese individuals have normal fasting glycemia with elevated plasma insulin, but become progressively more hyperglycemic and insulin resistant with a decline in plasma insulin levels associated with pancreatic β-cell dysfunction (2). Diabetic hyperglycemia results from a failure of insulin and elevated plasma glucose to increase glucose utilization and suppress endogenous glucose production (2). With >90% of endogenous glucose production derived from liver (3,4) and as much as 40% of alimentary glucose taken up by liver (5–7), for storage as glycogen (8–10), a progressive loss in these liver functions is associated with the deterioration of glycemic control and the eventual development of diabetes.

Whether liver uses or produces glucose is mostly determined by the activity of the first and last enzymes of hepatic glucose utilization and production, respectively. Net hepatic glucose flux is therefore the balance between the rate of glucose phosphorylation catalyzed by glucokinase (GK), the first step of hepatic glucose utilization, and the rate of glucose-6-phosphate (G-6-P) dephosphorylation catalyzed by glucose-6-phosphatase (G-6-Pase), the last step of hepatic glucose production. In studies of nondiabetic rats, glucose-induced suppression of net hepatic glucose production was associated with increased glucose phosphorylation (11) and GK activity was required for a rise in plasma glucose to suppress hepatic glucose production (12).

Male Zucker diabetic fatty (ZDF) rats are a widely used genetic model of obese type 2 diabetes, since many characteristic features of this model are common with human obese type 2 diabetes (13). Young ZDF rats exhibit normal fasting glycemia with slightly elevated plasma insulin levels and become progressively more hyperglycemic and insulin resistant as plasma insulin levels are decreased with pancreatic β-cell dysfunction (14). Previously, we reported that blunted hepatic glucose flux in response to a rise in plasma glucose and insulin, as seen in the early (10 weeks of age) and middle (14 weeks of age) phases of diabetes development in ZDF rats, is associated with impaired regulation of GK by GK regulatory protein (GKRP) (15–17). In our current study, we investigated GK expression in liver during the progressive development of diabetes in ZDF rats and examined what correlation altered GK expression may have with the development of defective hepatic glucose metabolism and hyperglycemia. We report here that GK expression in liver is progressively reduced with the development of hyperglycemia in ZDF rats and that normalizing liver GK expression restores plasma glucose to nearly normal levels by improving the responsiveness of hepatic glucose metabolism to alterations in blood glucose during this later phase of diabetes development in ZDF rats.
Metabolites in blood and tissue. Glycogen and G-6-P content in liver, plasma glucose, plasma free fatty acids (FFAs), plasma triglyceride, plasma insulin, plasma glucagon, blood lactate, and blood alanine were determined as previously described (15,16). [2-3H]- and [3-3H]glucose levels in plasma glucose and liver glycogen were determined by selective enzymatic detritiation of [2-3H]glucose (16). External standards of [2-3H]- and [3-3H]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 assay. Overall completion of detritiation of [2-3H]glucose was 97.2 ± 0.4%, while 99.8 ± 0.3% of [3-3H]glucose remained intact. The specific activity of [2-3H]- and [3-3H]glucose (dpm/μmol) in plasma glucose was determined by the method of Debodo et al. (21).

Enzyme activities. For GK and G-6-Pase activity measurements, 200 mg freeze-clamped livers were homogenized in 2 ml buffer containing 50 mMol/l HEPES, 100 mMol/l KCl, 1 mMol/l EDTA, 5 mMol/l MgCl2, and 2.5 mMol/l dithioerythritol (22). Homogenates were centrifuged at 100,000 g for 45 min to sediment the microsomal fraction. GK and G-6-Pase activities were assayed in the postmicrosomal and the microsomal fractions, respectively, as described previously (16). Glycogen synthase and phosphorylase activities in the liver were measured using the method described by Golden et al. (23).

Calculations. Rates of [2-3H]- and [3-3H]glucose–determined glucose turnover were calculated as the ratio of the rate of infusion of [2-3H]- and [3-3H]glucose (dpm · kg⁻¹ · min⁻¹) and the [2-3H] and [3-3H] specific activity in plasma glucose, respectively, according to the steady-state equations of Steele et al. (24). Rate of endogenous glucose production was determined as the difference between [3-3H]glucose turnover rate and the glucose infusion rate. To estimate the amount of glucose incorporated into hepatic glycogen via the direct pathway, [3-3H] incorporated into glycogen was divided by the plasma glucose [3-3H] specific activity, respectively. Glucose cycling is defined as input of extracellular glucose into the G-6-P pool followed by the return of plasma glucose–derived G-6-P back into the extracellular pool; therefore, glucose cycling rate was calculated as the difference between [2-3H]glucose turnover rate and [3-3H]glucose turnover rate. The in vivo flux through G-6-Pase was calculated as the sum of endogenous glucose production plus glucose cycling.

Statistical analysis. The data collected are expressed as means ± SE. The significance of the differences between groups was analyzed by Student’s t test. Differences were considered significant when P < 0.05.

RESULTS

Progression of diabetes and changes in hepatic GK protein and activity along with aging in ZDF rats. As the ZCL rats aged, their BMI tended to increase along with an increase in body weight (Fig. 1A and B). While plasma insulin and glucagon decreased and increased, respec-
with vehicle.
rats treated with AdvCMV-mutGK; light gray dots, ZDF rats treated
and D (Fig. 2) this reduced level until the study was terminated on day 6
levels gradually fell over a 4-day period and remained at
GK activity in liver (Fig. 2).
levels were similar (Fig. 1). At 10 weeks of age, plasma
GK protein and activity were higher at 7
ZCL rats, body weight, BMI, plasma insulin, plasma gluc-
activity (Fig. 1). After 10 weeks of age, plasma insulin and hepatic
GK protein and activity progressively decreased as plasma
levels rose.
Effect of the treatment with AdvCMV-mutGK on hepatic GK activity and metabolic profiles. Hepatic GK activity, metabolic profiles (plasma levels of glucose, insulin, glucagon, lipid, and gluconeogenic precursors), and hepatic glucose fluxes (glucose turnover, glucose cycling, endogenous glucose production, and G-6-Pase flux) in 20-week-old ZDF rats treated with AdvCMV-mutGK were similar to that of ZDF rats treated with vehicle, indicating that viral infection per se and expression of a catalytically inactive form of GK protein did not affect hepatic GK activity and metabolism.
Effect of the treatment with AdvCMV-GKL on hepatic GK activity, plasma glucose, and body weight. Treatment of ZDF rats with AdvCMV-GKL increased GK activity dose-dependently (Fig. 2A). When ZDF rats were treated with AdvCMV-GKL at 1 or $2 \times 10^{11}$ pfu, blood glucose levels gradually fell over a 4-day period and remained at this reduced level until the study was terminated on day 6 (Fig. 2B). Body weight was not altered by this increased GK activity in liver (Fig. 2C and D).
Metabolite profile of 20-week-old ZDF rats treated with AdvCMV-GKL before and during a hyperglycemic clamp. Figures 3 and 4 show a relationship between measured metabolic parameters and hepatic GK activity, as indicated by a smooth trend line for parameters measured before and during the clamp, respectively. The normal level of GK expression in a nondiabetic liver was determined by measuring GK activity in ZCL rats that underwent the same hyperglycemic clamp. The activity was determined to be $32.5 \pm 3.2$ mU/mg total protein (the activity range was from 28 to 40 mU/mg). ZDF rats treated with AdvCMV-GKL were divided into three groups for analytical comparison based on the level of hepatic GK activity (ZDF-L, lower than normal range, 4–18 mU/mg; ZDF-N, normal range, 21–55 mU/mg; ZDF-H, higher than normal range, 64–91 mU/mg) as determined at the end of the hyperglycemic clamp, and these groups were compared with a group of ZCL rats that also underwent the same hyperglycemic clamp. Table 1 shows the summary of the metabolic profiles of these four groups of rats and statistical analyses of these metabolic parameters. Before a hyperglycemic clamp, compared with ZCL rats, ZDF-L rats had similar plasma insulin and glucagon levels and similar blood alanine levels. Plasma FFA level tends to be higher. Blood lactate and plasma triglyceride levels were much higher. Plasma glucose levels, [$2^{-3}$H]glucose turnover rates (which represent G-6-Pase flux), [$3^{-3}$H]glucose turnover rates, and glucose cycling rates were much higher. Plasma glucose levels were reduced and reached near-normal levels as hepatic GK activity approached that of ZCL rats, and the restoration of nearly normal plasma glucose levels were associated with decreased [$2^{-3}$H]- and [$3^{-3}$H]glucose turnover. Additionally, a further increase in GK activity to about twice that found in ZCL livers did not further decrease plasma glucose levels, but instead, tended to increase [$2^{-3}$H]- and [$3^{-3}$H]glucose turnover. While blood lactate levels were slightly increased, blood alanine levels, plasma insulin, plasma glucagon, and plasma triglycerides did not change.
In ZCL rats during a hyperglycemic clamp, when plasma glucose, insulin, and glucagon levels were raised to that seen in ZDF-L rats, levels of blood lactate, but not blood alanine and plasma triglyceride, tended to rise to that seen in ZDF rats. Glucose turnover rates were increased threefold, and endogenous glucose production was completely suppressed. Glucose cycling rates were also markedly increased, and G-6-Pase flux was not changed significantly. During a hyperglycemic clamp in ZDF-L rats, measured parameters were not changed from that of the basal period. In ZDF rats treated with AdvCMV-GKL, in parallel with increased GK activity in the liver, plasma insulin and glucagon were not changed from basal levels during the glucose clamp. Blood lactate, but not blood alanine and plasma triglyceride, levels were raised. Glucose turnover rates and glucose cycling rates increased. Additionally, endogenous glucose production was reduced with a decrease in G-6-Pase flux.
At the end of the clamp period, as compared with ZCL rats, in ZDF-L rats, G-6-P content (Fig. 5A) was significantly lower and the amount of incorporated glucose into glycogen via the direct pathway was greatly reduced (Fig. 5C). Glycogen phosphorylase a activity was significantly higher, and in contrast, glycogen synthase 1 activity was significantly lower (Fig. 5D–F). In ZDF rats treated with AdvCMV-GKL, in parallel with increased GK activity, G-6-P content, glycogen content, and the amount of glucose incorporated into glycogen via the direct pathway were increased (Fig. 5 and Table 1). Total activity of glycogen synthase was not changed, but the active form of glycogen synthase was increased. Phosphorylation a activity was not
Hepatic GK expression decreases progressively during the development of diabetes in ZDF rats. An increase in GK activity in liver has been described in obese human subjects (25), obese hyperinsulinemic Zucker rats (26), and young obese ob/ob mice (27). In coincidence with these reports, ZDF rats have higher hepatic GK expression at 7 weeks of age when they are obese with euglycemia and hyperinsulinemia. GK expression then decreases progressively with the development of diabetes in ZDF rats, so that by 20 weeks of age, activity is only 30% of that found in nondiabetic ZCL rats. We demonstrated that normalization of hepatic GK activity in 20-week-old ZDF rats improves hepatic glucose flux at basal as well as when presented with a hyperglycemic challenge. The improved hepatic glucose flux restores blood glucose to normal levels. This demonstrates that altered hepatic glucose metabolism, responsible for hyperglycemia at this late stage of diabetes in this animal model, at least partly, resulted from decreased GK activity.

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DISCUSSION

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progression (7 and 10 weeks of age), which corresponds to a time when GK expression remains at normal levels (15,17). However, at around 14 weeks of age, GK expression is markedly reduced, and GK is predominantly localized to the cytoplasm with the abnormal residency of GKRP in the cytoplasm (16). These data suggest the possibility that the inappropriate localization of GK in the cytoplasm may increase GK degradation and thus eventually result and/or contribute to lower GK levels.

**Restoration of hepatic glucose flux responsiveness to changes in plasma glucose by normalization of hepatic GK expression.** Net hepatic glucose flux is the balance between glucose utilization as initiated by glucose phosphorylation to form G-6-P mediated by GK and glucose production as concluded by G-6-P dephosphorylation mediated by G-6-Pase. Therefore, a relative decrease in G-6-Pase flux and/or a relative increase in GK flux would lead to the observed decrease of net hepatic glucose production. The lack of suppression of endogenous glucose production in response to a hyperglycemic challenge in ZDF rats was associated with lower G-6-P content and glycogenesis via the direct pathway, suggesting lower glucose phosphorylation by GK. Normalization of GK expression restored suppression of endogenous glucose production in response to a rise in plasma glucose levels. The restoration was associated with restored glucose phosphorylation by GK, since hepatic G-6-P content, glucose cycling rate, and incorporation of glucose into glycogen via the direct pathway all increased. The restoration of glycogen synthesis by the direct pathway in response to a rise of plasma glucose was associated with a restored activation of glycogen synthase. It has been reported that increases in G-6-P through mass action and its allosteric regulatory effect on downstream enzymes in hepatocytes plays a major role in mediating the effects of GK overexpression on hepatic glucose metabolism, glycogen synthesis, and glycolysis (34).

Hepatic glucose production is predominantly via gluconeogenesis in ZDF rats (16). The rate of gluconeogenesis is controlled principally by the activities of several key enzymes, including G-6-Pase, fructose-1,6-bisphosphatase, and PEPCK. Increased G-6-Pase flux was observed in the liver of ZDF rats compared with ZCL rats. Interestingly, there was a significant decrease in G-6-Pase flux in parallel with normalized GK activity before and during a hyperglycemic clamp (Table 1). Under a hyperglycemic clamp, decreased G-6-Pase flux was observed despite increased content of G-6-P, a substrate of G-6-Pase, which suggests G-6-Pase activity was decreased. However, the $V_{max}$ and apparent $K_m$ of G-6-Pase activity was decreased. The restoration of hepatic glucose flux responsiveness to changes in plasma glucose by normalization of hepatic GK expression.
TABLE 1

|                | Clamp | Basal |
|----------------|-------|-------|
| **Hepatic glucose flux (molar glucose/liver)** |       |       |
| Glucose production (mmol/kg) | 2.49 | 2.50 |
| Glucose cycling (mmol/kg)      | 18     | 11   |
| Rate (mmol/kg/min)             | 4.6   | 3.2 |
| Blood Lactate (mmol/l)         | 0.59 | 0.59 |
| Triglyceride (mg/ml)           | 0.35 | 0.35 |
| Alanine (mmol/l)               | 1.91 | 1.92 |

Significant differences (p < 0.05) from the corresponding values of the ZCL group, from the ZDF-L group, and from basal values within the same group are indicated.

ZDF rats were studied and three groups based on the level of plasma glucose levels by ADVANGL are described. H-CL (control), H-L (low), T-CL (treated).
decreased $V_{\text{max}}$ of G-6-Pase activity was observed after a short period of refeeding nondiabetic rats, as measured from homogenates of livers freeze-clamped in situ but not in the microsomal fractions isolated from these liver homogenates, which may suggest that an inhibitor that is either highly labile or that is lost during the process of microwave isolation (35). During the last decade, several intermediates of metabolism, such as free fatty acids, acyl-CoA, $\alpha$-ketoglutarate (36,37), and lipid products of phosphatidylinositol 3-kinase (38) have been reported to alter G-6-Pase activity allosterically. In a future study, we hope to identify what intracellular factor(s) may mediate the decreased G-6-Pase activity that is associated with the restoration of GK expression.

**Relationship between increased glucose cycling and GK expression.** ZDF rats have an increased rate of an apparent futile cycle of glucose to G-6-P and back to glucose that is a characteristic of insulin resistance and hyperglycemia seen with type 2 diabetes (39–41). This increased glucose cycling appears to be associated with increased rates of phosphorylation of glucose and/or dephosphorylation of G-6-P. Increased glucose phosphorylation increases the intracellular concentration of G-6-P and the fractional contribution of plasma glucose to this G-6-P pool. This also leads to an increased dephosphorylation of G-6-P derived from plasma glucose by increasing total G-6-P flux and/or the fraction of G-6-P derived from plasma glucose in the total G-6-P dephosphorylated by G-6-Pase (the later could occur even without an increase in total G-6-Pase flux).

It is unlikely that increased glucose cycling in ZDF rats is associated with only altered GK expression. Compared with ZCL rats, glucose cycling under a basal condition is threefold higher at an early stage of diabetes in ZDF rats (10 weeks of age), when these ZDF rats have similar GK expression, but do have lower GK activity due to a defective activation of the enzyme by glucose via an apparent failure of GK to dissociate from its inhibitory protein (GKRP) (15,17). Glucose cycling is increased further during the progression of diabetes in ZDF rats, as is found at 20 weeks of age when GK expression is decreased (Table 1) (15,16). However, the restoration and increase of GK expression at this phase of diabetes in ZDF rats did not affect glucose cycling. These findings suggest that other factors, other than lower GK activity, is involved in the increased glucose cycling associated with diabetes.

**Maintenance of GK expression in liver as a possible gene therapy–based approach for reducing hyperglycemia in type 2 diabetes.** O’Doherty et al. (42) reported that lowering of blood glucose and plasma insulin by a sixfold increase in hepatic GK expression in nondiabetic rats using an adenovirus were accompanied by marked increases in plasma triglycerides, FFAs, cholesterol, $\beta$-hydroxybutyrate, and lactate. Our study shows that increase in hepatic GK expression above normal tended to worsen hyperlipidemia and hyperlactemia in ZDF rats, a model of type 2 diabetes associated with obesity. We previously reported that >90% of hepatic glucose production in ZDF rats is due to gluconeogenesis (16). Since elevated GK expression decreased endogenous glucose production under basal conditions and during hyperglycemic clamp, it is probable that the observed increase in blood lactate levels associated with greater GK expression resulted from an inhibition of gluconeogenesis. Increased glycolytic flux and/or decreased gluconeogenic flux, as a consequence of GK overexpression, would likely increase the concentra-
tion of substrates for de novo lipogenesis and esterification of triglyceride. Furthermore, excessively increased G-6-P concentration would stimulate expression of the fatty acid synthase gene via activation through its carbohydrate response element (43,44). Therefore, an excessive induction of hepatic GK expression might have a risk to generate or worsen complications associated with type 2 diabetes.

On the other hand, O’Doherty et al. (42) also reported that a mild (threefold) increase in hepatic GK expression in nondiabetic rats did not alter the basal metabolic profile. Transgenic mice overexpressing GK exhibited a slight decrease in blood glucose levels and no alteration of plasma lipid levels (45). These observations suggested a less impact of mildly increased hepatic GK expression on basal glycemia and lipemia in normal animals. Nevertheless, transgenic mice expressing GK at approximately twofold of that in the wild-type mice are more tolerant to diet–induced diabetes (47,48). These benefits are not accompanied by altering plasma lactate, FFAs, and triglycerides (46–48). Our present study shows in 20-week-old ZDF rats that a short-term normalization of GK expression in liver improves hepatic glucose flux and restores blood glucose levels to near normal, even at the late stage of diabetes where plasma glucose levels are >25 mmol/l and compensatory hyperinsulinemia does not exist anymore. It has been recently reported that single-dose administration of GK activator lowers fasting and postglucose–challenged plasma glucose levels by declining endogenous glucose production, in addition to by improving glucose utilization in patient with type 2 diabetes (49). These results clearly suggest that maintenance of near-normal GK activity in the liver, either by a gene therapy–based approach or possibly by pharmaceutical intervention, is a likely therapeutic target for the restoration of normoglycemia in type 2 diabetes.

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