Glucokinase Overexpression Restores Glucose Utilization and Storage in Cultured Hepatocytes from Male Zucker Diabetic Fatty Rats

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Joan Seoane†‡, Albert Barberà†‡, Sabine Télémaque-Potts‡, Christopher B. Newgard‡, and Joan J. Guinovart†‡**

From the †Department of Biochemistry and Molecular Biology, University of Barcelona, E-08028 Barcelona, Spain and the ‡Gifford Laboratories for Diabetes Research, Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Zucker diabetic fatty rats develop type 2 diabetes concomitantly with peripheral insulin resistance. Hepatocytes from these rats and their control lean counterparts have been cultured, and a number of key parameters of glucose metabolism have been determined. Glucokinase activity was 4.5-fold lower in hepatocytes from diabetic rats than in hepatocytes from healthy ones. In contrast, hexokinase activity was about 2-fold higher in hepatocytes from diabetic animals than in healthy ones. Glucose-6-phosphatase activity was not significantly different. Despite the altered ratios of glucokinase to hexokinase activity, intracellular glucose 6-phosphate concentrations were similar in the two types of cells when they were incubated with 1–25 mM glucose. However, glycogen levels and glycogen synthase activity ratio were lower in hepatocytes from diabetic animals. Total pyruvate kinase activity and its activity ratio as well as fructose 2,6-bisphosphate concentration and lactate production were also lower in cells from diabetic animals. All of these data indicate that glucose metabolism is clearly impaired in hepatocytes from Zucker diabetic fatty rats.

Glucokinase overexpression using adenovirus restored glucose metabolism in diabetic hepatocytes. In glucokinase-overexpressing cells, glucose 6-phosphate levels increased. Moreover, glycogen deposition was greatly enhanced due to the activation of glycogen synthase. Pyruvate kinase was also activated, and fructose 2,6-bisphosphate concentration and lactate production were increased in glucokinase-overexpressing diabetic hepatocytes. Overexpression of hexokinase I did not increase glycogen deposition. In conclusion, hepatocytes from Zucker diabetic fatty rats showed depressed glyco-gen and glycolytic metabolism, but glucokinase overexpression improved their glucose utilization and storage.

Non-insulin-dependent diabetes mellitus (NIDDM) is associated with insulin secretory defects that occur together with insulin resistance (1–3). The insensitivity to insulin and relative insulin deficiency in NIDDM lead to a decrease in glucose utilization by liver, muscle, and adipose tissue and to an increase in the hepatic glucose production (4). Neither the role of hepatic metabolism in the development of type 2 diabetes nor its alterations are well established.

The development of diabetes in male Zucker diabetic fatty rats (ZDF rats) has many features in common with human NIDDM (5). Male ZDF rats develop progressive insulin resistance and glucose intolerance between 3 and 8 weeks of age and usually become completely diabetic between 8 and 10 weeks, whereas their lean counterparts (ZLC rats) do not develop either insulin resistance nor diabetes. Therefore, they are considered a genetic model of type 2 diabetes. ZDF rats are hyperinsulinemic throughout development.

In mammalian cells, glucose is phosphorylated to glucose 6-phosphate (Glu-6-P) by members of the hexokinase family. Hepatocytes from healthy rats contain mainly hexokinase IV, usually called glucokinase (GK), and small amounts of hexokinases I, II, and III. GK differs from the rest of hexokinases in that it is approximately half as large, it is not allosterically inhibited by Glu-6-P, and it has a much higher $K_{\text{m}}$ for glucose (8 mM versus 50–100 $\mu$M). In recent studies, we have shown that overexpression of GK, but not hexokinase I, in healthy Wistar rat hepatocytes by adenovirus-mediated gene transfer leads to marked enhancement of glycolysis and glycogen synthesis (6, 7). Other studies in transgenic mice showed the same effect of GK overexpression in vivo and concluded that its overexpression can correct hyperglycemia in streptozotocin-treated mice, a model of IDDM (8, 9).

We studied various parameters of glucose metabolism in cultured hepatocytes from ZDF rats and compared them with those determined from hepatocytes isolated from ZLC rats. We show that hepatocytes from Zucker diabetic fatty rats have a depressed glycolysis and glucose metabolism, but glucokinase overexpression improves their glucose utilization and storage.

**Experimental Procedures**

Preparation of Recombinant Adenoviruses—Recombinant adenoviruses containing the cDNA encoding rat liver GK (AdCMV-GKL) and HK I (AdCMV-HKI) were prepared as described previously (10, 11).

Hepatocyte Isolation, Culture, and Transfection with Recombinant Adenoviruses—Recombinant adenoviruses containing the cDNA encoding rat liver glucokinase; AdCMV-GKL, recombinant adenovirus containing the cDNA of rat liver glucokinase; AdCMV-HKI, recombinant adenovirus containing the cDNA of hexokinase I.
**Glucokinase Overexpression on ZDF Rat Hepatocytes**

**Adeno-virus—Male ZDF (ZDF/Gmi-fa/fa) and ZLC (ZLC/+fa and +/+) rats** were purchased from Genetic Models (Indianapolis, IN). The animals were used when they were 12–13 weeks old. At this age, the blood glucose levels (9:00–10:00) were 24 ± 4 mm in ZDF and 5.2 ± 0.5 mm in ZLC rats. Hepatocytes were isolated from 24-h fasted animals by collagenase/enzyme digestion as described (12). Cells were suspended in Dulbecco’s modified Eagle’s medium (Whittaker), 100 mM insulin (Roche Molecular Biochemicals), and 100 mM dexamethasone (Sigma) and seeded onto plastic plates of 60-mm diameter treated with 0.1% gelatin (Sigma) at a final density of 6 × 10^6 cells/cm^2. After cell attachment (5 h at 37 °C), hepatocytes were treated with stocks of AdCMV-GKL or AdCMV-HKI at a multiplicity of infection of 10 for 2 h at 37 °C.

After treatment with the virus, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 1 mM glucose, 1 mM dexamethasone, and 1 mM insulin. Cells were incubated for 16 h, and then experimental manipulations were performed as detailed in the text and figure legends. At the end of each experimental manipulation, cell monolayers were frozen in liquid N_2 until analysis.

**Enzyme Activity Assays**—To measure enzyme activities, 100 μl of homogenization buffer consisting of 10 mM Tris-HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 600 mM sucrose, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride was added to frozen plates containing the cell monolayers, and cells were collected with a plastic scraper. Cells were lysed by thawing and checked under light microscopy for completeness. Homogenates were collected in Eppendorf tubes and centrifuged at 10,000 × g for 15 min at 4 °C, and supernatants and pellets were used for determinations.

Protein concentration was measured as described by Bradford (13) using the Bio-Rad assay reagent. Glucose-phosphorylating activity was measured spectrophotometrically in 10,000 × g supernatant fractions of hepatocyte extracts at 37 °C in the presence of 100 mM and 0.5 mM glucose as described (14). The phosphorylating capacity obtained at 0.5 mM glucose is considered the hexokinase activity, while the subtraction of the activity measured at 0.5 mM glucose from the activity measured at 100 mM glucose is considered the glucokinase activity of the extract. 

Glycogen synthase activity was measured in the presence or absence of 6.6 mM Glu-6-P in both supernatant and pellet fractions as described (15). The activity measured in the absence of Glu-6-P represents the active form of the enzyme (or a form), whereas the activity tested in the presence of 6.6 mM Glu-6-P is a measure of total activity. The ratio of these two activities is known as the glycogen synthase activity ratio and is an estimate of the extent of the activation of the enzyme. Glucose-6-phosphatase activity was measured in both supernatant and pellet fractions as described (16).

Pyruvate kinase (PK) activity was measured spectrophotometrically. Frozen plates were scraped with 100 μl of a homogenization buffer at pH 7.4 with 50 mM glycglycine, 15 mM EDTA, 100 mM KF, and 5 mM potassium phosphate. The homogenates were centrifuged at 10,000 × g for 15 min at 4 °C, and total PK activity and activity ratio (V_{P/K} to V_{P/K}) were determined in the supernatants as described (17).

**Metabolite Determinations**—Glycogen content was measured by scraping cells into 300 μl of a homogenization buffer at pH 7.4 with 50 mM glycglycine, 15 mM EDTA, 100 mM KF, and 5 mM potassium phosphate. The homogenates were centrifuged at 10,000 × g for 15 min at 4 °C, and the content was determined as described (18). The total activities of Glu-6-P were measured by spectrophotometric assays (20).

**L-Lactate levels in the incubation medium** were measured as described (21).

**Glycolysis in Hepatocytes from ZDF and ZLC Rats**

To determine the glycolytic flux of the hepatocytes from ZLC and ZDF rats, lactate production, PK activity, and Fru-2,6-P_2 concentration were analyzed. Lactate accumulation was measured using an experimental approach identical to that described earlier for the determination of Glu-6-P. We found a sharp glucose-dependent increase in lactate production and in Fru-2,6-P_2 concentration in hepatocytes treated with ZDF rats, but not in hepatocytes from ZDF rats (Fig. 2). Lactate production in hepatocytes from ZDF rats was higher than in hepatocytes from ZDF rats at all glucose concentrations tested. Hepatocytes from ZDF rats hardly modified their lactate production in response to an increase in glucose concentration, and only a slight increase in Fru-2,6-P_2 concentration was observed.

**Table I**

| Glucokinase | Hexokinase | Glucose-phosphatase | Pyruvate kinase |
|-------------|------------|---------------------|----------------|
| ZDF         | 16.2 ± 1.7 | 3.8 ± 0.6           | 20.3 ± 2.4     |
| ZLC         | 3.5 ± 0.4* | 6.5 ± 0.9*          | 19.6 ± 1.9     |

* p < 0.001 compared with ZLC cells.

**p < 0.01 compared with ZLC cells.**
Glycogen Synthesis in Hepatocytes from ZDF and ZLC Rats

To evaluate glycogen synthesis in ZDF and ZLC hepatocytes, a number of assays were performed. We determined the glycogen content using an experimental approach identical to that described earlier for the determination of Glu-6-P levels in ZLC hepatocytes. A glucose concentration-dependent increase in glycogen content in ZLC hepatocytes was found. In contrast, ZDF hepatocytes deposited only negligible amounts of glycogen in response to glucose (Fig. 1). In light of this dramatic difference, we determined the activity of the rate-limiting enzyme for glycogenesis, glycogen synthase. Total glycogen synthase activity, measured in the presence of 6.6 mM Glu-6-P, was not significantly different in ZDF and ZLC hepatocytes (Table I). The PK activity ratio was also lower in ZDF than in ZLC hepatocytes (0.20 ± 0.03 versus 0.31 ± 0.04). This activity ratio was not altered when cells were incubated in different glucose concentrations (Fig. 3). These results could explain the difference in lactate accumulation observed in ZDF hepatocytes in comparison with ZLC hepatocytes. PK is one of the rate-limiting steps in glycolysis; therefore, a decrease in its activity is clearly reflected in the overall flux of glycolysis.

Glycogen Synthesis in Hepatocytes from ZDF and ZLC Rats

We have previously shown that GK overexpression greatly enhances glucose utilization and glycogen deposition in hepatocytes from healthy rats (6, 7). It was then interesting to study the impact of GK overexpression in hepatocytes of this genetic model of NIDDM and evaluate the possibility that GK overexpression could correct the metabolic alterations observed in ZDF rat hepatocytes.

**Glucokinase Overexpression in ZDF Hepatocytes—**The efficiency of the recombinant adenovirus for overexpression of GK in rat hepatocytes is well documented (6, 7). AdCMV-GKL-treated ZDF hepatocytes showed a GK activity of 168 ± 19 milliunits/10⁶ cells. This value was 48- and 10-fold higher than in untreated ZDF and ZLC hepatocytes, respectively. Untreated and AdCMV-GKL-treated ZDF hepatocytes preincubated for 16 h in 1 mM glucose and then transferred to media containing 1, 5, 10, and 25 mM glucose for 2 h showed a glucose concentration-dependent increase in Glu-6-P levels (Fig. 5). AdCMV-GKL-treated cells had higher levels than untreated hepatocytes in all conditions studied, but the effect was more pronounced at higher glucose concentration. The maximal difference was observed at 25 mM glucose, where Glu-6-P intracellular concentration was 15-fold higher than in ZDF-untreated cells.

**Effect of GK Overexpression in ZDF Hepatocytes on Glycolysis—**In order to evaluate the effect of GK overexpression in ZDF hepatocytes on the glycolytic flux, lactate production, PK activity, and Fru-2,6-P₂ concentration were determined. AdCMV-GKL-treated cells produced about 3-fold more lactate and had 6–7-fold more Fru-2,6-P₂ than untreated ZDF in all conditions studied.
Glucokinase Overexpression on ZDF Rat Hepatocytes

Effects of GK overexpression on Glu-6-P levels and glycogen content in ZDF hepatocytes. ZDF hepatocytes were treated with AdCMV-GKL (●) or left untreated (○) and incubated for 16 h in the presence of 1 mM glucose. Cells were then exposed to the concentrations of glucose indicated for 2 h and collected for measurement of intracellular Glu-6-P levels (A) and glycogen content (B). Data represent the mean ± S.E. for five independent experiments. *, p < 0.01; ***, p < 0.001, compared with untreated ZDF cells.

Effect of GK Overexpression in ZDF Hepatocytes on Glycogen Synthesis—Glycogen levels were 9–13-fold higher in GK-overexpressing hepatocytes than in untreated cells in all conditions studied (Fig. 5). Total glycogen synthase activity was not significantly different in GK-overexpressing and untreated ZDF hepatocytes (2.1 ± 0.1 milliunits/10⁶ cells versus 2.2 ± 0.2 milliunits/10⁶ cells), and the amount of glycogen synthase protein measured by immunoblotting was also the same in the two groups (data not shown). However, in GK-overexpressing ZDF hepatocytes, the capacity of glycogen synthase to be activated in a glucose-dependent manner in both supernatant and pellet fractions was restored. The glycogen synthase (~Glu-6-P/ +Glu-6-P) activity ratio, determined in the 10,000 × g supernatant and pellet fractions, was much higher in AdCMV-GKL-treated cells than in untreated ZDF cells (Fig. 6, A and B). The maximal difference in the activity ratio was observed at 25 mM glucose in supernatants (0.46 ± 0.02 versus 0.17 ± 0.02) and in pellets (0.48 ± 0.03 versus 0.16 ± 0.02). The higher activation state of glycogen synthase in AdCMV-GKL-treated ZDF hepatocytes explains their enhanced capacity to synthesize glycogen in comparison with untreated ZDF hepatocytes.

Metabolic Effects of HK I Overexpression in Hepatocytes from ZDF Rats

In our previous work (6, 7), we observed that HK I overexpression did not affect glycogen deposition in hepatocytes from healthy rats. We thus attempted to determine whether HK overexpression affects glycogen in hepatocytes from ZDF rats. The efficiency of AdCMV-HKI for overexpression of HK I in rat hepatocytes is well known (6, 7). AdCMV-HKI-treated ZDF hepatocytes showed a 4-fold increase in HK activity. Glycogen levels were not significantly different in HK I-overexpressing hepatocytes and untreated cells (Table II) at any of the concentrations of glucose tested.

Discussion

ZDF rats develop type 2 diabetes concomitantly with peripheral insulin resistance. Although a good deal of information about the β-cell function of this model is available, much less is known about the carbohydrate metabolism in the liver of these animals. In this study, we have analyzed the response to glucose of cultured hepatocytes from ZDF rats in comparison with ZLC hepatocytes, their nondiabetic counterparts. ZDF hepatocytes clearly showed a decrease in their capacity to store glucose as glycogen and a fall in glycolytic flux. These result in a low rate of glucose utilization, which is a characteristic pattern of a diabetic phenotype. These defects are probably a consequence of the low activity of GK and PK in these cells. Decreased GK and PK activities have also been observed in the liver of ZDF rats, and these alterations are preserved when hepatic cells are isolated and cultured. This indicates that ZDF cultured hepatocytes are a good model to study the alterations of carbohydrate metabolism in these animals.

In the liver, glucose is stored as glycogen. The synthesis of this polysaccharide is a process that contributes to control of glycemia in the postprandial state. NIDDM is associated with an impairment of glycogen synthesis, which results in a decrease of glucose utilization by the liver, contributing to hyperglycemia. ZDF rat hepatocytes show a clear decrease in the ability to synthesize glycogen, although they have no alteration in total glycogen synthase activity. As described (22–24), glucose induces the activation of liver glycogen synthase through an increase in Glu-6-P, which causes the covalent activation of glycogen synthase, thus leading to an increase in glycogen accumulation. Although ZDF and ZLC hepatocytes show similar increases in the intracellular levels of Glu-6-P when incubated with glucose, glycogen synthase is normally activated in ZLC, while it remains essentially inactive in ZDF rat hepatocytes. A critical observation is that GK activity is very low in ZDF cells, while conversely HK activity is higher. Therefore, in ZLC hepatocytes Glu-6-P is mainly produced by GK, whereas in ZDF cells glucose phosphorylation is catalyzed mainly by other hexokinases. These data provide further support for our recent proposal of a differential regulatory impact of Glu-6-P produced by hexokinase versus GK in liver cells (7). Since

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Glu-6-P is mainly produced by hexokinase in ZDF hepatocytes, this metabolite is not able to trigger activation of glycogen synthase and for this reason glucose does not induce glycogen synthesis. All of these results suggest that GK plays a key role in the activation of glycogen synthase and that the lack of GK activity is most probably responsible for the deficiency in glycogen synthesis of ZDF hepatocytes.

Further support for this hypothesis was obtained by GK overexpression in ZDF hepatocytes. The increase in the intracellular levels of Glu-6-P produced by GK in the diabetic cells led to the activation of glycogen synthase and to an increase in glycogen accumulation, as we have observed in healthy hepatocytes (7). Therefore, GK overexpression is able to rescue the ability of ZDF cells to synthesize glycogen in response to glucose. In clear agreement with our previous results (6, 7) overexpression of HK I was without effect on glycogen deposition.

On the other hand, glycolytic flux is also decreased in ZDF hepatocytes. Lactate production is much lower in ZDF hepatocytes than in ZLC hepatocytes. This result can be explained by the observed deficit in total and active PK activity and the decrease in the levels of Fru-2,6-P2. This metabolite controls a key step of glycolysis regulating the activity of phosphofructose kinase-1 and phosphofructokinase-1. PK is also one of the limiting steps of glycolysis. PK activity is 5–6-fold lower in ZDF hepatocytes than in ZLC hepatocytes, and, moreover, its activation state is also lower.

GK-overexpressing cells showed an increased glycolytic flux. Lactate production was 3-fold higher in ZDF hepatocytes treated with AdCMV-GKL. This result can be explained by the sum of several factors. First, the increase in GK activity produced a great increase in Glu-6-P levels that exerted a push effect and also resulted in an increase in the levels of Fru-2,6-P2, which raised the flux through phosphofructokinase-1. Second, in GK-overexpressing cells, PK total activity as well as its activation state were increased. This increase in PK total activity can be explained by an enhancement in PK gene transcription by Glu-6-P as described in Refs. 25 and 26. The net result of all of these factors would be an increase of the rate of lactate production.

In summary, GK overexpression induced an increase in glucose utilization both by triggering glucose storage into glycogen and by increasing glycolysis. This finding indicates that GK overexpression can overcome the deficiency in glucose utilization characteristic of the hepatocytes isolated from ZDF rats and suggests gene therapy strategies that may be relevant to type 2 diabetes. In normal fasted animals, the liver has a net glucose synthesis and output, and after feeding it switches to net glucose uptake and storage. In type 2 diabetes, the liver fails to respond to postprandial increases in glucose. Studies in a variety of animal models of diabetes have led to the suggestion that lowering of the GK/glucose-6-phosphatase enzyme activity ratio, whether by a decrease in GK, an increase in glucose-6-phosphatase, or a combination of the two, may impair hepatic glucose metabolism and contribute to the etiology of diabetes (27–29). In our model of type 2 diabetes, ZDF rats, we also observed a decrease in the GK/glucose-6-phosphatase ratio caused by the significant decrease in GK activity. Our results suggest that GK supplementation in ZDF rats may

**Table II**  
**Effects of GK and HK I overexpression on glycogen content in ZDF hepatocytes**  
ZDF hepatocytes were treated with AdCMV-GKL and AdCMV-HKI or left untreated and incubated for 16 h in the presence of 1 mM glucose. Cells were then exposed to the concentrations of glucose indicated for 2 h and collected for measurement of glycogen content. Data represent the mean ± S.E. for five independent experiments.

| Glucose (mM) | Untreated ZDF | AdCMV-HKI ZDF | AdCMV-GKL ZDF |
|-------------|---------------|---------------|---------------|
| 1           | 6.6 ± 1       | 7.2 ± 0.7     | 13.0 ± 0.86   |
| 5           | 9.1 ± 0.9     | 7.2 ± 1.6     | 45.7 ± 1.74   |
| 10          | 11.5 ± 0.9    | 10.2 ± 0.2    | 94.5 ± 1.3    |
| 25          | 17.6 ± 0.8    | 18.8 ± 1      | 102.2 ± 11.4  |

In conclusion, the observed deficits in total and active PK activity and the decrease in Fru-2,6-P2 levels suggest that GK overexpression can overcome the deficit in glucose utilization characteristic of the hepatocytes isolated from ZDF rats.
enhance hepatic glucose clearance, possibly to an extent that will result in a decrease in circulating glucose concentration. However, a cautionary note in this regard comes from a recent study in normal rats in which adenovirus-mediated overexpression of glucokinase in liver resulted in lowering of blood glucose, but with accompanying increases in free fatty acid and triglyceride levels (30). It remains to be determined whether similar complications will arise when glucokinase is overexpressed in liver of NIDDM models such as the ZDF rat.

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