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Long-term overexpression of glucokinase in the liver of transgenic mice leads to insulin resistance

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Abstract

Aims/hypothesis. Glucokinase overexpression in the liver increases glucose uptake and utilization, and improves glucose tolerance in young transgenic mice. Here, we examined the long-term effects of hepatic overexpression of glucokinase on glucose homeostasis. Moreover, we determined whether glucokinase overexpression counteracted high-fat diet-induced insulin resistance.
Methods. Transgenic mice overexpressing glucokinase in liver under the control of the phosphoenolpyruvate carboxykinase promoter, fed either a standard diet or a high-fat diet, were studied. We used non-transgenic littermates as controls.
Results. Transgenic mice over 6 months old developed impaired glucose tolerance. In addition, at 12 months of age, transgenic mice showed mild hyperglycaemia, hyperinsulinaemia and hypertriglyceridaemia. In spite of increased glucokinase activity, the liver of these mice accumulated less glycogen and increased triglyceride deposition. When 2-month-old glucose-tolerant mice were fed a high-fat diet, transgenic mice gained more body weight and became hyperglycaemic and hyperinsulinaemic. This was concomitant to glucose intolerance, liver steatosis and whole-body insulin resistance.
Conclusion/interpretation. Long-term overexpression of glucokinase increases hepatic lipogenesis and circulating lipids, which lead to insulin resistance. Our results also suggest that the liver plays a key role in the onset of diabetes. [Diabetologia (2003) 46:1662–1668]

Keywords Transgenic mice, glucokinase, high-fat diet, glucose metabolism, insulin resistance, Type 2 diabetes.

Glucokinase (GK) is the main glucose-phosphorylating enzyme in the liver and pancreatic beta cells. Glucose transport and phosphorylation are the first steps in glucose utilization in the liver, where GK contributes to glucose disposal. Several studies in vitro have shown that GK activation is needed for glycolysis and glycogen synthesis [1, 2, 3, 4]. Similarly, in transgenic mice overexpressing GK in the liver, intracellular glucose 6-phosphate, glycogen and L-pyruvate kinase activity are increased, indicating that the activation of GK can induce glycolysis and glycogen synthesis in vivo [5]. These transgenic mice have reduced glycaemia, insulinaemia and blood glucose after an intraperitoneal glucose tolerance test, indicating that GK overexpression increased blood glucose disposal by the liver [5]. Similar results have also been obtained in transgenic mice expressing the human GK gene in the liver [6] and in mice overexpressing one or more extra copies of the entire mouse GK gene locus [7].

Since the decrease in glucokinase gene expression in diabetic animals could contribute to hyperglycaemia, the effect of hepatic GK overexpression has been studied in animal models of Type 1 and Type 2 diabetes. Thus, streptozotocin-treated GK-expressing transgenic mice maintained normal levels of GK activity,

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Abbreviations: GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase.

which were associated with increased glycolysis and reduced gluconeogenesis. This leads to normalization of blood glucose in the absence of insulin [8]. Furthermore, a high-fat diet did not induce diabetes in transgenic mice overexpressing one copy of the GK gene [9]. In these mice, hepatic GK overexpression reduced hyperglycaemia and hyperinsulinaemia induced by a high-fat diet. Moreover, adenovirus-mediated expression of GK in the liver improves glucose tolerance and reduces hyperinsulinaemia in high-fat diet-induced diabetes in mice [10]. These results point to overexpression of GK as a possible gene therapy-based approach to reduce hyperglycaemia in diabetes.

However, high levels of adenovirus-mediated GK overexpression decrease blood glucose concentrations with concomitant increases in circulating free fatty acids and triglycerides [11]. Moreover, an increase in GK activity has been described in obese-diabetic subjects [12] and in obese hyperinsulinaemic (*fa/fa*) Zucker rats [13], suggesting that hepatic GK overexpression could have diabetogenic effects. Therefore, the aim of this study was to examine the long-term effects of GK overexpression in the liver of transgenic mice expressing a *PEPCK/GK* chimeric gene. We found that old transgenic mice with increased GK activity developed glucose intolerance and hepatic lipid accumulation. In addition, these mice were more sensitive to high-fat diet-induced diabetes. These results suggest that GK overexpression might exacerbate diabetes and confirm the key role of the liver in its onset.

Materials and methods

Treatment of mice. Transgenic mice expressing the GK gene in liver under the control of the PEPCK promoter in a C57Bl6;SJL mixed genetic background were used [5]. The line of transgenic mice with the highest copy number and activity were used in this study [5]. We used littermates as controls for the transgenic animals. Mice were fed ad libitum with a standard diet (Panlab, Barcelona, Spain) and maintained under a light-dark cycle of 12 h (lights on at 8:00 a.m.). For the experimental induction of Type 2 diabetes, mice were kept in individual cages and fed a high-fat diet (TD 88137, Harlan Teklad Laboratory, Madison, Wis., USA) for up to 12 weeks. Where stated, mice were starved for 16 h. Animals were anaesthetized with isofluorane (between 9 and 11 a.m.), killed by decapitation and blood and tissue samples obtained. In the experiments described below, heterozygous male mice aged 8 weeks at the start of the diet were used. All experimental procedures involving mice were approved by the Ethics and Experimental Animal Committee of the Universitat Autònoma de Barcelona.

Enzyme, metabolite and hormone assays. To measure GK activity, liver samples were homogenized in an ice-cold buffer (pH 7.4) containing 50 mmol/l Tris-HCl, 300 mmol/l sucrose, 100 mmol/l KCl, 1 mmol/l ethylenediamine tetraacetic acid, and 0.7 μl/ml β-mercaptoethanol. GK activity was analyzed in 12 000×*g* supernatants [14] and was calculated as the difference between the glucose phosphorylation capacity at 100 mmol/l and 0.5 mmol/l glucose. To measure the concentration of metabolites, liver samples were immediately frozen in

liquid N_2. The concentrations of hepatic glycogen, and glucose 6-phosphate were measured in perchloric extracts, which were adjusted to pH 5 with 5 M K_2CO_3 for glycogen, and to pH 7 for glucose 6-phosphate. Glycogen levels were measured by the α-amyloglucosidase method [15], and glucose 6-phosphate was determined enzymatically [16]. Hepatic triglyceride content was determined by extracting total lipids from liver samples with chloroform-methanol (2:1 vol/vol) as described by another study [17] separating the chloroform and methanol-water phases. Triglycerides were then quantified spectrophotometrically using an enzymatic assay kit (GPO-PAP, Roche Molecular Biochemicals, Mannheim, Germany). Glucose was measured enzymatically (Glucoquant®, Roche) in serum. Glucose was also determined in 5 μl of blood by using Glucometer® Elite analyzer (Bayer Diagnostics Europe, Dublin, Ireland). Serum triglycerides were determined enzymatically (GPO-PAP, Roche, Germany). Serum insulin concentrations were measured by radioimmunoassay (CIS, Biointernational, Gif-Sur-Yvette, France), using rat insulin (Eli Lilly, Indianapolis, Ind., USA) as standard.

Glucose and insulin tolerance tests. For glucose tolerance test, awake control and transgenic mice fasted overnight (16 h) with free access to water were given an intraperitoneal injection of glucose (1 g/kg of body weight). Blood samples were obtained from the tail vein before the glucose injection and at the indicated time points after the glucose load, and glucose concentration was measured. For insulin tolerance test, insulin (0.75 IU/kg body weight; Humulin regular, Eli Lilly) was injected intraperitoneally into awake, fed control and transgenic mice. Glucose concentration was determined in blood samples obtained from the tail vein at the indicated time points after the insulin injection.

Histological analysis. The livers from control and transgenic mice were fixed for 12 to 24 h in formalin, embedded in paraffin and sectioned. Sections were stained with haematoxylin/eosin.

Statistical Analysis. Enzyme activities, serum parameters and metabolite concentrations are expressed as the means±SEM. Analysis of statistical differences between data were carried out using the Student-Newmann-Keuls test. A *p* value of less than 0.05 was considered statistically significant.

Results

Long-term overexpression of GK in the liver led to insulin resistance. To examine whether long-term overexpression of GK affected glucose disposal, an intraperitoneal glucose tolerance test was carried out in transgenic mice at different ages (Fig. 1). In contrast to 2-month-old transgenic mice, which showed increased glucose disposal, 3-month-old transgenic animals showed no improvement in glucose tolerance compared with control animals (Fig. 1A). Furthermore, 6-month-old transgenic mice reached higher blood glucose concentrations than control mice and did not recover basal glucose at 180 min after glucose administration (Fig. 1B). Similar results were observed in 12-month-old transgenic mice (data not shown). This indicates that GK overexpression leads to progressive, age-dependent glucose intolerance.
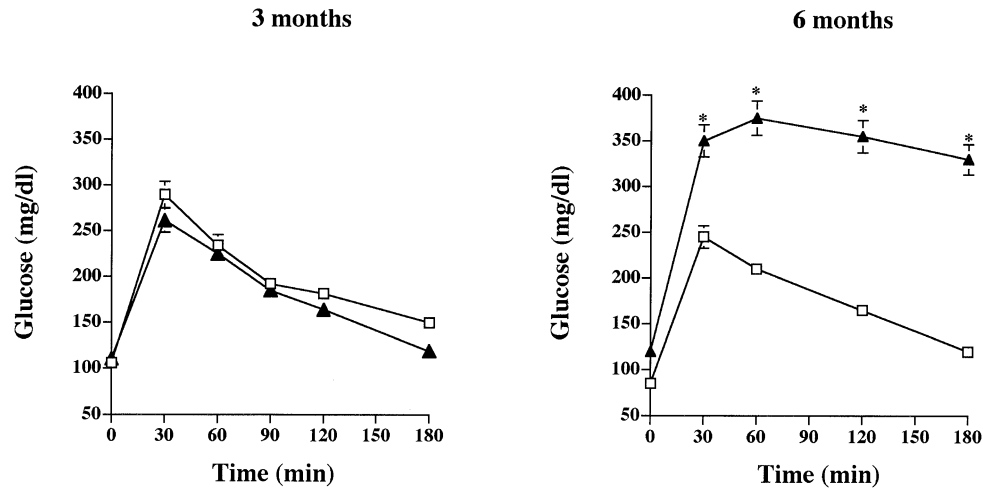
3 months

6 months

Fig. 1. Intraperitoneal glucose tolerance test. Overnight-starved transgenic (▲) and control (□) (3- and 6-month-old) mice were given an i.p. injection of 1 mg of glucose per g body weight.

Blood samples were taken from the tail vein of the same animals at the times indicated. Results are means±SEM of 8 transgenic and 10 control mice. * $p<0.05$ vs. control mice
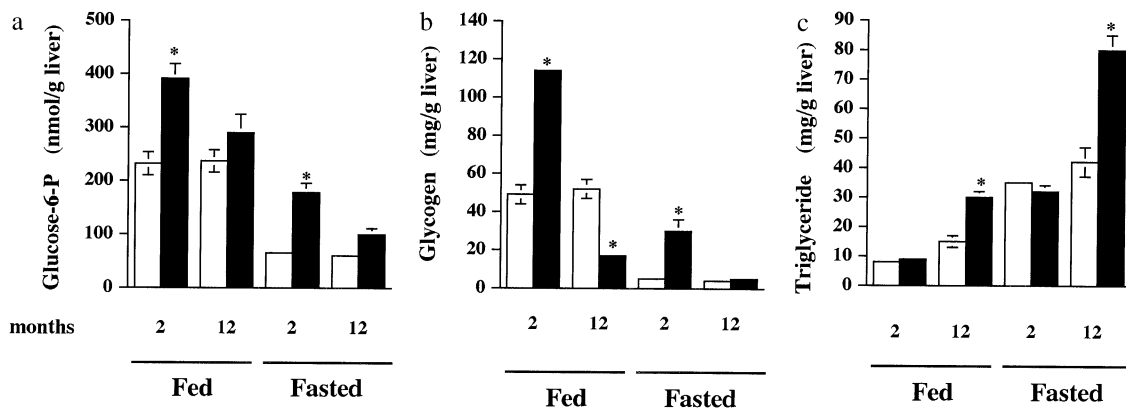

Fig. 2a–c. Glucose-6-P, glycogen and triglyceride content in the liver of transgenic mice expressing glucokinase. The concentration of **a**: glucose 6-phosphate, **b**: glycogen and **c**: triglyceride was measured in liver extracts obtained from fed and fasted control and transgenic mice. Results are means±SEM of 18 different animals in each group. * $p<0.05$ vs. control mice

Table 1. Serum parameters in fasted transgenic mice overexpressing GK

	Glucose (mg/dl)	Insulin (ng/ml)	Triglycerides (mg/dl)
Control	88±3	1.5±0.2	120±2
Transgenic	132±4*	3.3±0.7*	203±2*

Glucose, insulin and triglycerides in serum of control and transgenic mice were determined. Results are ± SEM of at least 15 different animals in each group. * $p<0.05$ vs. control mice

Table 2. Liver glucokinase activity

	Glucokinase (mU/mg prot)	
	2 months	12 months
Control	4±2	5±3
Transgenic	19±2*	10±3*

Glucokinase activity was measured in liver extracts of fasted control and transgenic mice. Results are the means±SEM of 12 transgenic mice from each line and 10 control mice. * $p<0.05$ vs. control mice

Furthermore, in contrast to 2-month-old fasted transgenic mice, 12-month-old mice presented mild hyperglycaemia, hyperinsulinaemia (about two-fold increase) and an increase in triglyceride levels (about 1.6-fold) compared with control mice (Table 1). These findings suggested that transgenic mice developed insulin resistance with aging. Since GK was under the control of the PEPCK promoter, GK activity was determined in fasted mice. 2-month-old transgenic mice showed higher (about five-fold) GK activity than controls (Table 2). However, 12-month-old animals showed only a two- fold increase in GK activity. Thus, GK activity decreased in transgenic mice when older.

2-month-old transgenic mice accumulate more glycogen and glucose-6-P in the liver than controls both in fed and fasted conditions (Fig. 2A,B). 12-month-old fed control and transgenic mice showed similar

concentrations of glucose-6-P. However, fasted transgenic mice showed an increase in glucose-6-P that was parallel with the increase in GK activity (Fig. 2A). In spite of similar concentrations of glucose-6-P in transgenic and control mice, 12-month-old fed transgenic mice showed a marked decrease (about 70%) in hepatic glycogen content (Fig. 2B). Upon fasting, glycogen storage in both control and transgenic animals was nearly completely mobilized. To determine whether the decrease in glycogen storage was parallel to increased lipid metabolism, intrahepatic triglyceride content was measured in old animals. In fasted and fed conditions, hepatic triglyceride concentration was higher in transgenic mice (about 1.5 and two-fold respectively) than in controls (Fig. 2C). All these results suggested that transgenic mice showed altered hepatic glucose and lipid metabolism.
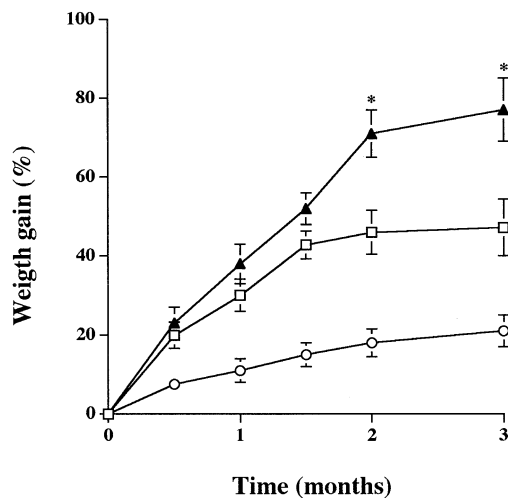
Transgenic mice overexpressing GK were not protected from diet-induced obesity and insulin resistance. After 3 months on a high-fat diet, transgenic mice had gained about 80% body weight whereas control mice only gained 40% (Fig. 3). In contrast, control mice fed a standard diet showed an increase of about 15% in body weight (Fig. 3). Although transgenic mice had increased body weight, food intake was similar to that of controls (3.5 ± 0.1 and 3.3 ± 0.1 g/day for control and Tg mice). Thus, overexpression of GK did not protect mice from diet-induced obesity. After the feeding of a high-fat diet, control mice were mildly hyperglycaemic and hyperinsulinaemic (about three-fold increase) compared to controls fed a standard diet. Transgenic mice showed a similar increase in glycaemia to controls (Fig. 4A). However, transgenic mice had an increase in serum insulin levels (about six-fold compared to control mice fed a standard diet and about two-fold compared to control mice fed a high-fat diet) (Fig. 4B). In addition, when an intraperitoneal glucose tolerance test was carried out after 8 weeks in a high-fat diet, transgenic mice reached higher blood glucose concentrations than controls and did not recover basal glucose at 180 min. Moreover, blood glucose concentrations in control mice were higher than those of control mice fed a standard diet (Fig. 5A).

Whole-body insulin sensitivity of transgenic mice was measured after 2.5 month on a high-fat diet by an insulin tolerance test. In fat-fed transgenic mice, the hypoglycaemic effect of insulin was abolished while fat-fed control mice showed a reduced response to insulin compared to control mice fed a standard diet (Fig. 5B). This indicated that transgenic mice were more insulin resistant than controls when fed a high-fat diet. Histological analysis of liver sections from mice fed a high-fat diet for 3 months showed dramatic vacuolization due to fat deposition (Fig. 6). Although control mice showed mild lipid accumulation, transgenic mice developed liver steatosis. All these results indicate that transgenic animals under a high-fat diet showed an exacerbated diabetic phenotype.

Fig. 3. Effect of a high-fat diet in body weight gain of transgenic mice overexpressing GK in liver. Changes in body weight of control mice fed a standard diet (○), and control (□) and transgenic (▲) mice fed a high-fat diet for 3 months are shown. Data are means±SEM of 12 mice for each group. *$p<0.05$ Tg-fat vs. Con-fat
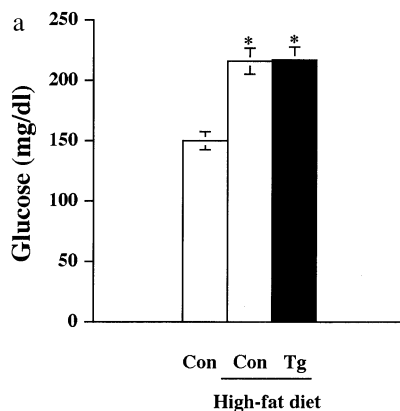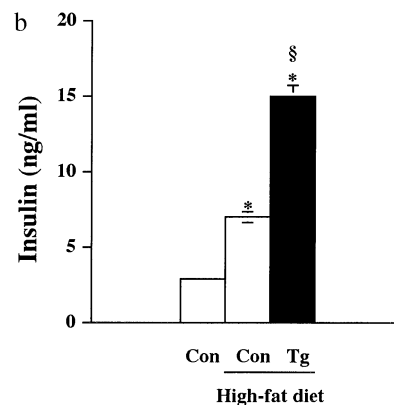

Fig. 4a, b. Serum insulin and blood glucose concentrations. **a**: Glucose and **b**: Insulin were measured in control mice fed a standard diet and in control and transgenic mice after 3 months on a high-fat diet in fed conditions. Results are means±SEM of 12 animals for each group. *$p<0.05$ vs. control mice; § $p<0.05$ Tg-fat vs. Con-fat
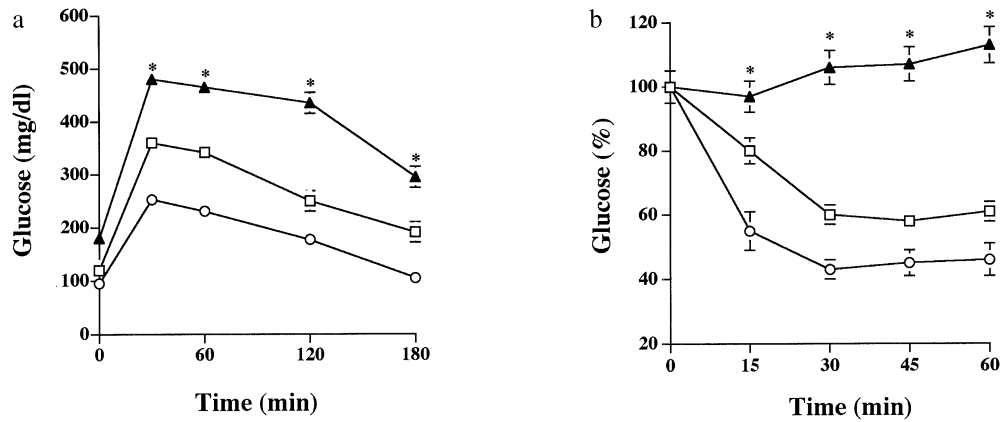
Fig. 5a, b. Intraperitoneal glucose and insulin tolerance tests. **a**: Intraperitoneal glucose tolerance test. Awake overnight-starved control fed a standard diet (○) and control (□) and transgenic (▲) mice after 2.5 months on a high-fat diet were given an intraperitoneal injection of glucose (1 mg/g of body weight). Blood samples were taken at the times indicated from the tail vein of the same animal. Results are means±SEM of 12 animals for each group. *p<0.05;Tg-fat vs. Con-fat. **b**: Intra-peritoneal insulin tolerance test. Insulin was injected intraperitoneally into awake control fed a standard diet (○), and control (□) and transgenic (▲) fed a high-fat diet. Blood samples were taken from the tail vein of the same animals at the time indicated and glucose levels were determined. Results were calculated as percentage of blood glucose values at time 0 for each mouse and are means±SEM of six animals for each group. *p<0.05; Tg-fat vs. Con-fat
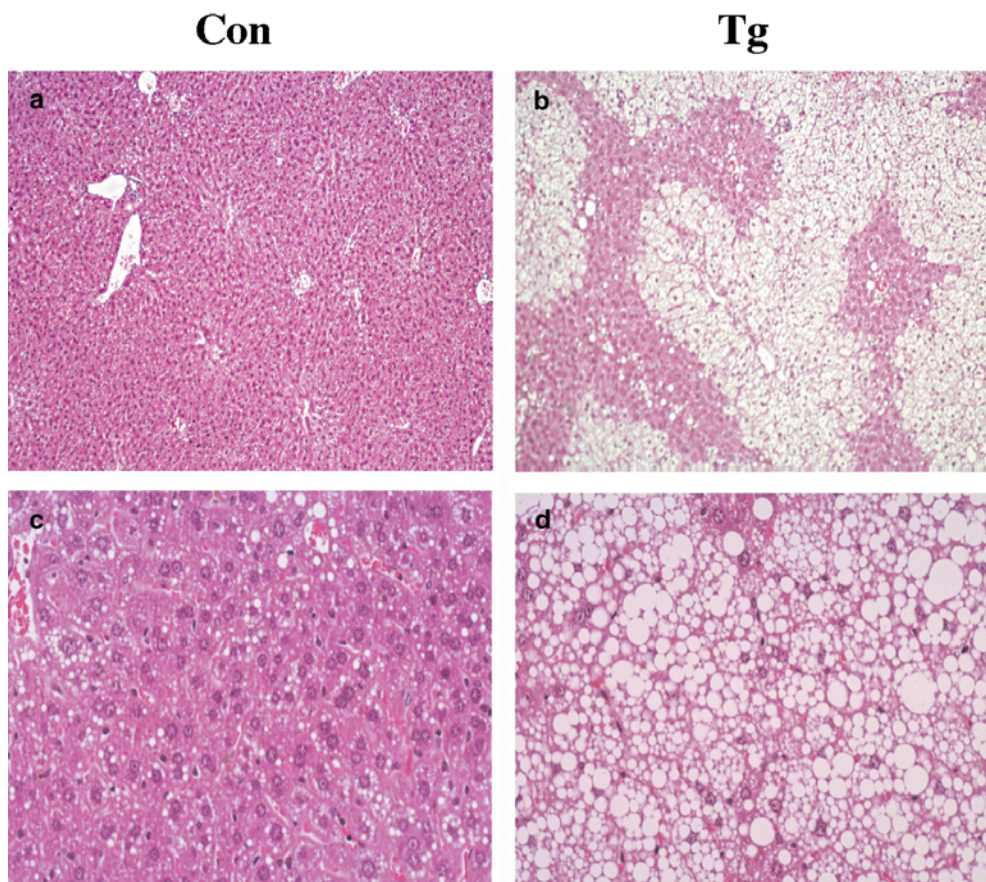

Fig. 6a–d. Long-term overexpression of GK leads to fatty liver. Representative sections of the liver from control (**a**, **c**) and transgenic Tg (**b**, **d**) mice stained with haematoxylin and eosin are presented. (Upper panel ×100 and bottom panel ×400)

Discussion

Short-term increase in hepatic GK expression increases glycolytic flux and glycogen storage [1, 5, 6, 7]. A previous report has shown that a short-term adenovirus-mediated six-fold-increase in GK activity lowers blood glucose concentrations at the cost of an increase in circulating triglyceride and FFA levels [3]. In contrast, a slight increase in GK activity (three-fold) had no effect on glycaemia or serum triglyceride levels [3]. Here we showed that long-term GK overexpression leads to hyperglycaemia, hyperinsulinaemia, hepatic steatosis and increased serum triglyceride levels, resulting in glucose intolerance and lower insulin sensitivity. Thus, our results together with these other findings indicate that GK overexpression has deleterious effects depending on the level and the length of the increase in GK activity.

Transgenic mice overexpressing GK fed a high-fat diet gained more body weight than controls and became glucose intolerant and insulin resistant. In contrast, overexpression of one copy of the GK gene slightly lowers hyperglycaemia and hyperinsulinaemia induced by a high-fat diet [9]. However, these mice also overexpressed GK in beta cells [18] whereas in the present study, transgenic mice specifically overexpressed GK in the liver. Thus, differences between the two transgenic models could be due to the level of hepatic transgene expression and the tissue-specificity.

Type 2 diabetes is characterized by glucose metabolism alterations such as failure of insulin to inhibit hepatic gluconeogenesis and impaired skeletal muscle glucose uptake [19]. However, lipid metabolism is also altered. This is often reflected by increased circulating free fatty acids and triglycerides together with increased fat accumulation in non-adipose tissues [20, 21]. Thus, changes in the equilibrium between glucose and fatty acid metabolism in liver and muscle could be responsible for glucose homeostasis alterations. It has been shown that an increase in glucose metabolism leads to higher hepatic malonyl-CoA levels, which in turn inhibit carnitine palmitoyl transferase 1 (CPT1) activity, resulting in a decrease of long chain fatty acyl CoA transport into the mitochondria and a decrease of fatty acid oxidation [22, 23]. Consequently, the fat content of the liver and VLDL secretion increase, thus contributing to the development of hypertriglyceridaemia. This, in turn, could lead to triglyceride accumulation in skeletal muscle which might contribute to peripheral insulin resistance. In agreement with this, GK overexpression leads to an increase of fat deposition in the liver and circulating triglyceride levels in transgenic mice. This probably occurred through an increase of fat synthesis from glucose and/or a decrease of fatty acid oxidation due to CPT1 inhibition. The resulting long-chain fatty acyl CoA cytosolic accumulation could inhibit glucose metabolism, thus contributing to hyperglycaemia observed in the transgenic animals. Moreover, the increased fat deposition probably decreases hepatic insulin extraction, contributing to hyperinsulinaemia in our transgenic mice [24]. The decrease in glycogen content observed in these mice could be due to hepatic insulin resistance. This is consistent with the negative correlation observed between hepatic glycogen and triglyceride content in rats fed a high-fat diet [25]. Thus, our results suggest a strong interaction between glucose and lipid metabolism and show that a disruption in the equilibrium between the two pathways might lead to insulin resistance.

In humans, fat accumulation in the liver is associated with hepatic insulin resistance, fasting hyperinsulinaemia and hypertriglyceridaemia [26]. Moreover, adipose tissue deficiency in lipodistrophy is associated with hepatic steatosis, increased circulating lipids and insulin resistance [27, 28]. Insulin sensitivity can be restored in a mouse lipodistrophic model by fat transplantation, probably by recovering the capacity of adipose tissue to accumulate lipids and reducing hepatic fat [29]. These findings together with our results suggest that increased lipid deposition in the liver could result in hyperglycaemia and impaired glucose tolerance.

All these results indicate that a primary alteration in liver might lead to whole-body metabolic alterations. This has also been shown in insulin receptor-deficient mice. Thus, while skeletal muscle has been postulated to be the major site of insulin resistance, mice lacking insulin receptor in muscle have normal whole-body glucose homeostasis [30]. In contrast, liver-specific insulin receptor-deficient mice showed hyperinsulinaemia, hyperglycaemia, insulin resistance and impaired glucose tolerance [31]. Moreover, phosphoenolpyruvate carboxykinase overexpression in the liver leads to increased chronic hepatic glucose production and then to increased insulin secretion, hyperinsulinaemia, hyperglycaemia and insulin resistance [32, 33, 34, 35]. In agreement with this, our results suggest that alteration in hepatic glucose metabolism could be an initial event in the diabetic process. In summary, this study indicates that long-term overexpression of GK results in increased hepatic triglyceride content and circulating lipid levels, which leads to insulin resistance and Type 2 diabetes. Furthermore, our results suggest that gene therapy approaches based on overexpressing GK in the liver for diabetes treatment should consider the side effects of increased GK activity.

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