Animal Model for Maturity-onset Diabetes of the Young Generated by Disruption of the Mouse Glucokinase Gene*

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Glucokinase catalyzes a rate-limiting step in glucose metabolism in hepatocytes and pancreatic β cells and is considered the "glucose sensor" for regulation of insulin secretion. Patients with maturity-onset diabetes of the young (MODY) have heterogeneous point mutations in the glucokinase gene that result in reduced enzymatic activity and decreased insulin secretion. However, it remains unclear whether abnormal liver glucose metabolism contributes to the MODY disease. Here we show that disruption of the glucokinase gene results in a phenotype similar to MODY in heterozygous mice. Reduced islet glucokinase activity causes mildly elevated fasting blood glucose levels. Hyperglycemic clamp studies reveal decreased glucose tolerance and abnormal liver glucose metabolism. These findings demonstrate a key role for glucokinase in glucose homeostasis and implicate both islets and liver in the MODY disease.

Pancreatic β cells and hepatocytes are the two major cell types responsible for maintaining glucose homeostasis. β cells respond to changes in plasma glucose levels by regulating their insulin release, whereas hepatocytes adjust their glucose uptake and glucose production to changes in plasma insulin. Both cell types express a specialized high Km member of the hexoki-nase family of enzymes, glucokinase (GK), which catalyzes a rate-limiting step in glucose metabolism, the phosphorylation of glucose to glucose 6-phosphate. In β cells glucose metabolism generates signals for insulin secretion, and GK is considered the major "glucose sensor" that couples the extracellular glucose levels to insulin secretion (1). Recent DNA polymorphism studies of patients with maturity-onset diabetes of the young (MODY), a form of non-insulin-dependent (type II) diabetes mellitus, have established that heterozygous point mutations in the GK gene are associated with the development of diabetes (2, 3). The mutations result in reduced enzymatic activity (4, 5), which causes abnormal glucose sensing and decreased insulin secretion (6–8). In β cells the GK promoter is constitutively active, and GK activity is regulated by glucose at post-transcriptional levels (9). The dominance of the GK mutations in MODY has therefore been explained as a gene dosage effect. In contrast, hepatocytes utilize a different GK promoter, which is regulated by insulin (10, 11). This promoter has the potential to up-regulate the expression of the normal allele in hepatocytes of MODY patients to compensate for the reduced GK activity caused by the mutant allele. However, this transcriptional regulation depends on plasma insulin levels, which in MODY patients are reduced due to the islet abnormality. Hence, it remains unclear whether abnormal liver glucose metabolism contributes to the MODY disease. Previously we have attenuated GK function specifically in β cells in transgenic mice using an antisense approach (12). These mice manifested a decreased insulin secretory response to glucose; however, they did not show changes in fasting plasma glucose levels or glucose tolerance. Here we used homologous recombination in mouse embryonic stem cells to assess the effects of disrupted GK function in both β cells and hepatocytes and generate an animal model for MODY.

MATERIALS AND METHODS

Generation of Mutant GK Mice—A fragment of the glucokinase gene was cloned from a Ch35A Mbol partial 229/Sv genomic library (Stratagene, La Jolla, CA) using a 5‘-labeled rat GK cDNA probe. The location of the exons was confirmed by DNA sequencing. Portions of this fragment were then used to construct a positive-negative targeting vector (13). Twenty-five μg of linearized plasmid DNA were electroporated into 2 × 10⁷ E14 ES cells, and the cells were grown and selected as described (13). G418- and gangcyclovir-double-resistant clones were screened for homologous recombination by PCR using the sense primer CGCTTACAAACTGGTGATT and the antisense primer GAAAGCTTAGACACAGGTAG. PCR consisted of 20 cycles of 1 min each at 94 °C and 58 °C, and 2 min at 72 °C, followed by 15 additional cycles with a 5-s autoextension. One out of 9 clones was positive, as further confirmed by Southern blot analysis. Four targeted cell clones were selected for injection into C57BL/6 blastocysts, which gave rise to 25 chimeric mice. Chimeric male mice were bred to C57BL/6 females, and agouti offspring were tested for germline transmission of the mutation by PCR and PCR with Southern blots. One male out of 14 was capable of germline transmission. Heterozygous GK +/− males and females were mated, and the offspring were analyzed by Southern blot analysis for the presence of the targeted and the endogenous GK locus.

In vivo Glucokinase Activity—Islets were isolated from 8-week-old male mice as described (14). Liver samples were obtained following the hyperglycemic clamp studies as detailed below. The tissues were homogenized and assayed for glucose phosphorylation activity by a fluorescent method as described (9). Vmax values are determined by Eadie-Hofstee plots with the best-fitted lines drawn by the method of least squares.

Plasma Glucose and Insulin—Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Inc., Palo Alto, CA). Plasma insulin was quantitated by a radioimmunoassay using a rat insulin standard.
Hyperglycemic Clamp—Four- to 6-month-old male mice (28–35 g) were anesthetized with chloral hydrate (400 mg/kg of body weight, intraperitoneally), and an indwelling catheter was inserted into the right internal jugular vein as described previously (15–17). Mice were studied 4–6 days post-surgery in the awake, unrestrained state using the hyperglycemic clamp technique in combination with infusion of high performance liquid chromatography-purified [3-3H]glucose (Du-Pont NEN; 10 μCi bolus, 0.1 μCi/ml) as described previously (15). Mice were fasted for 6 h before the infusion. A variable infusion of a 25% glucose solution was started at time zero and periodically adjusted to clamp the plasma glucose concentration at 15–18 mm. The studies lasted 170 min and included an 80-min basal period for assessment of basal turnover rates and a 90-min hyperglycemic clamp period. Plasma samples for determination of [3H]glucose specific activity were obtained from the tail vein at 40, 60, 70, and 80 min during the basal period and at 40, 60, 70, 80, and 90 min during the clamp period. Steady-state conditions for the plasma glucose concentration and specific activity were achieved within 40 min in both the basal and clamp periods of the studies. Plasma samples for determination of plasma insulin concentrations were obtained at 40, 0, 40, 60, 70, 80, and 90 min. The total volume of blood drawn was ~0.9 ml; to prevent volume depletion and anemia, 1.2 ml of fresh blood (obtained by heart puncture from a littermate of the test animal) and heparinized saline (10 units/ml) (1:1, v/v) was infused during the study. At the end of the glucose infusion, mice were anesthetized with 60 mg/kg pentobarbital intravenously, the abdomen was quickly opened, portal vein blood obtained, and the liver freeze-clamped in situ with aluminum tongs preceded in liquid nitrogen. The time from the injection of the anesthetic until freeze-clamping of the liver was less than 45 s. All tissue samples were stored at ~80 °C for subsequent analysis. Plasma [3H]glucose radioactivity was measured in duplicate in the supernatants of Ba(OH)2 and ZnSO4 precipitates (Somogy procedure) of plasma samples (25 μl) after evaporation to dryness to eliminate tritiated water (16, 17). Under steady-state conditions the rate of glucose disappearance (Rd) equals the rate of glucose appearance (Rb). The latter was calculated as the ratio of the rate of infusion of [3-3H]glucose (dpm/min) and the steady-state plasma [3H]glucose specific activity (dpm/μmol). Hepatic UDP-glucose (UDPG) concentration and specific activity in the liver were obtained through two sequential chromatographic separations, as reported previously (16, 17).

RESULTS AND DISCUSSION

A targeting vector was constructed in which a fragment of the mouse GK gene spanning exon 2 was replaced with a neomycin resistance gene, resulting in a deletion and frameshift in the transcript (Fig. 1A). This vector was used to disrupt the GK gene in embryonic stem (ES) cells and generate mice heterozygous for the mutation (Fig. 1B).

Analysis of glucose phosphorylation activity in islet homogenates of GK +/+ mice revealed a 37% reduction compared with GK +/+ littermates (Table I). This value is less than the expected 50% reduction in activity and may represent compensation by increased transcription of the wild-type allele, increased translation or stability of the wild-type GK mRNA, or by post-translational mechanisms. Liver GK activity in GK +/+ mice was reduced by 28% compared with GK +/+ controls (Table I). Based on the documented inducibility of the liver GK promoter by insulin (10, 11), the wild-type allele could be expected to fully compensate for the reduced GK activity caused by the mutant allele. However, the liver GK activity observed in the GK +/+ mice represents only partial compensation. There were no statistically significant differences in activity of the ubiquitous hexokinase in both islets and liver of GK +/+ compared with control mice (Table I).

Plasma glucose levels in fed GK +/+ mice were normal; however, overnight fasting levels were 25% higher than those of GK +/+ littermates (Table II). Thus, the reduced GK activity induces mildly elevated fasting glucose levels in the GK +/+ mice, as is the case in MODY patients (6–8). Plasma insulin
Glucose phosphorylation activity in GK +/- mice

Values are mean ± S.E. (n = 5–8) of V_{max} expressed as units/g (1 unit = 1 μmol of product/min at room temperature). GK values are significantly different between GK +/- and +/− by t test (p < 0.05), while HK values are not. HK, hexokinase.

| Genotype | Islets | Liver |
|----------|--------|-------|
| GK +/+   | 2.16 ± 0.11 | 0.77 ± 0.06 |
| GK +/-   | 1.37 ± 0.25 | 1.06 ± 0.14 |

Plasma glucose and insulin levels in GK +/- mice

Male mice (5–6 months old) were bled following an overnight fast or under fed conditions. Values are mean ± S.E. (n = 10). Fasted glucose levels are significantly different between GK +/+ and +/- by t test (p < 0.05), while fed glucose levels, and fed and fasted insulin levels, are not.

| Genotype | Glucose | Insulin |
|----------|---------|---------|
|          | Fasted | Fed |
|          | Fasted | Fed |
| GK +/+   | 3.6 ± 0.2 | 7.1 ± 0.4 | 4.4 ± 1.02 | 24.28 ± 3.07 |
| GK +/-   | 4.5 ± 0.3 | 6.7 ± 0.6 | 2.99 ± 0.47 | 18.27 ± 4.57 |

Hyperglycemia inhibits hepatic glucose production (HGP) and stimulates hepatic glucose uptake and glycogen synthesis. This process requires enhanced phosphorylation of plasma glucose in hepatocytes, resulting in increased flux through GK. An impairment in hepatic glucose phosphorylation capacity may cause impaired hepatic adaptation to changes in plasma glucose concentrations. These parameters were assessed by infusing [3-3H]glucose during the hyperglycemic clamp studies and determining HGP and hepatic UDPG formed from plasma glucose. Hyperglycemia and hyperinsulinemia reduced HGP by 72% in GK +/+ mice and by only 47% in GK +/- mice (Table III). The relative contribution of plasma glucose to liver glycogen repletion was evaluated by calculating the ratio of the specific activities of hepatic [3H]UDPG and portal vein plasma glucose, which provides a measure of the contribution of plasma glucose to the hepatic UDPG pool (“direct” pathway), as opposed to UDPG generated by gluconeogenesis. As shown in Table III, the contribution of the direct pathway to hepatic UDPG was markedly decreased in GK +/- mice compared with GK +/+ mice. A significant correlation (r = 0.47; p < 0.05) was demonstrated between the GIR during the hyperglycemic clamp studies and the fraction of hepatic UDPG derived from the direct pathway in the two groups combined. A modest
decrease in liver GK activity in the GK +/− mice is sufficient to affect hepatic glucose metabolism. These findings suggest that abnormalities in liver glucose metabolism contribute to the MODY disease.

Breeding of heterozygous mutants failed to generate GK −/− mice. Approximately one third of the embryos in timed pregnancies were found to be resorbed starting at E9.5, indicating that the absence of GK is lethal to embryos. At this stage, only primordial buds of liver and exocrine pancreas without islets are observed. Nevertheless, RT-PCR analysis of total RNA from wild-type E9.5 embryos revealed significant GK expression (Fig. 3), and E9.5 embryo extracts contained GK enzymatic activity (data not shown). Breeding of GK mutant mice with a transgenic mouse line expressing a GK transgene rescued the homozygous mutant, demonstrating that the embryonic lethality results from the absence of GK activity. These findings suggest an important function for GK during development; however, the cell types in which its activity is required remain to be identified.

In conclusion, disruption of one allele of the GK gene is associated with decreased tolerance to glucose and moderate impairments in both hepatic and β-cell glucose sensing. Thus a partial reduction in mouse islet and liver GK activity causes a disease very similar to human MODY, underscoring the key role of GK in maintaining glucose homeostasis. In contrast, a β-cell specific reduction of GK activity in transgenic mice, which results in a decreased insulin secretory response to glucose, is not sufficient to cause detectable changes in fasting plasma glucose levels or glucose tolerance (12). These findings indicate that the MODY disease results from abnormal glucose metabolism in both islets and liver. The GK +/− mice will allow studies on genetic and environmental factors that may interact with the impaired GK activity to cause overt diabetes.

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