Effects of Increased Glucokinase Gene Copy Number on Glucose Homeostasis and Hepatic Glucose Metabolism*

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The relationship between glucokinase (GK) gene copy number and glucose homeostasis was studied in transgenic mice with additional copies of the entire GK gene locus (Niswender, K. D., Postic, C., Jetton, T. L., Bennett, B. D., Piston, D. W., Efrat, S., and Magnuson, M. A. (1997) J. Biol. Chem. 272, 22564–22569). The plasma glucose concentration was reduced by 25 ± 3% and 37 ± 4% in mice with one or two extra copies of the gene locus, respectively. The basis for the hypoglycemic phenotype was determined using metabolic tracer techniques in chronically cannulated, conscious mice with one extra GK gene copy. Under basal conditions (6-h fasted) transgenic mice had a lower blood glucose concentration (−12 ± 1%) and a slightly higher glucose turnover rate (+8 ± 3%), resulting in a significantly higher glucose clearance rate (+21 ± 2%). Plasma insulin levels were not different, suggesting that increased glucose clearance was due to augmented hepatic, not islet, GK gene expression. Under hyperglycemic clamp conditions the transgenic mice had glucose turnover and clearance rates similar to the controls, but showed a lower plasma insulin response (−48 ± 5%). Net hepatic glycogen synthesis was markedly elevated (+360%), whereas skeletal muscle glycogen synthesis was decreased (−40%). These results indicate that increased GK gene dosage leads to increased hepatic glucose metabolism and, consequently, a lower plasma glucose concentration. Increased insulin secretion was not observed, even though the transgene is expressed in islets, because hypoglycemia causes a down-regulation in islet GK content (Niswender, K. D., Postic, C., Jetton, T. L., Bennett, B. D., Piston, D. W., Efrat, S., and Magnuson, M. A. (1997) J. Biol. Chem. 272, in press).

Glucokinase (GK) is thought to be the principal determinant of both hepatic and islet glucose utilization (2). The prediction, over a decade ago, that diminished GK activity would cause diabetes (3) has been validated with the discovery of GK gene mutations as the cause of maturity onset diabetes of the young, type 2 (MODY-2) (4, 5) and by the generation of GK gene knock-out mice (6–8). The loss of one functional GK allele leads to an early onset, but relatively mild hyperglycemia that is due, at least in part, to decreased activity of GK in the pancreatic β cell. This causes an increase in the glucose set point and attenuates glucose-stimulated insulin secretion (6–10). Less is known about the role of altered hepatic GK gene expression in MODY-2, even though GK activity has long been thought to be a key determinant of hepatic glucose usage (2). Studies using heterozygous GK null mice have been helpful, but only provide insights into the effects of diminished GK expression. For this reason, the effects of increased GK gene expression in the liver have also been studied in both transgenic mice and primary hepatocytes. In transgenic mice, increased hepatic GK causes hypoglycemia (11, 12), whereas in primary hepatocytes increased GK leads to both increased glycolysis and glycogen synthesis (13, 14).

Both the liver and islet, key sites of GK gene expression, participate in a feedback loop that is necessary for maintaining euglycemia (15, 16). The differential, tissue-specific regulation of GK gene expression is thought to be an essential component of this feedback loop (16). The liver can be either a net consumer or net producer of glucose, as determined in large part by the concentrations of insulin and glucagon in the portal blood (17–19), as well as the plasma glucose level itself. In turn, the secretion of pancreatic hormones depends on the plasma glucose concentration, which is affected in part by the status of the liver. In liver, GK gene transcription is stimulated by insulin and inhibited by glucagon (20, 21). In addition, GK activity in liver can be acutely inhibited through interactions with the GK regulatory protein (GKRP) (22–24). In the islet, GK gene transcription is thought to be largely constitutive, although glucose modulates islet GK content, probably by directly affecting the half-life of the enzyme (25, 26).

Metabolic flux in the liver reflects the net activities of several pathways. Some of the rate-determining enzymes in these pathways are regulated at the transcriptional level by the metabolism of glucose itself, presumably through a glycolytic intermediate (27). Carbohydrate response elements have been identified in the genes for L-type pyruvate kinase (L-PK) (28–30), S14 (31, 32), fatty acid synthase (33, 34), and GLUT2 (35). The induction of hepatic genes by glucose has been found to require GK, suggesting that GK gene expression has both catalytic and regulatory functions in the liver. Given the complex and interdependent factors involved in the regulation of both islet and hepatic GK, it is difficult to accurately predict the in vivo ramifications of altered GK gene expression from in vitro model systems.

To further understand the effect of physiologically relevant variations in GK gene copy number on glucose homeostasis, we
studied whole body glucose metabolism in mice containing a GK gene locus transgene (1). Using this experimental design, we were able to determine the effect on the plasma glucose concentration and hepatic glucose metabolism of one and two additional GK gene copies. These studies clearly revealed the reciprocal relationship between GK gene copy number and blood glucose concentration, the high control strength that GK gene expression has on blood glucose homeostasis, and the importance of feedback interactions between the liver and islet.

EXPERIMENTAL PROCEDURES

**GK Gene Locus Transgenic Mice**

The animals used in this study contain an 83-kilobase pair GK gene locus transgene inserted by pronuclear DNA microinjection into B6D2 F1 hybrid mice. Previous studies have shown there is one copy of the transgene per haploid genome, located on the X chromosome (1). All studies were performed using female animals from line 37 that were either heterozygous or homozygous for the transgene allele. Controls were non-transgenic animals of similar genetic background generated from within the same mouse colony. Only non-obese animals weighing 25–30 g were studied. Mice were housed either in a barrier facility or individual filtered units, fed standard mouse chow (Purina Mills, Inc.), and maintained on a 12-h light/dark cycle.

**Glucose Tolerance Test**

Mice were anesthetized with sodium pentobarbital (70 μg/g body weight), and blood was sampled from the retro-orbital sinus for glucose and hematocrit determination and analyzed immediately. A 50% glucose solution, infused at a variable rate, was used to maintain hyperglycemia in the hyperglycemic clamp studies. In total, anemia. A 50% glucose solution, infused at a variable rate, was used to

**Animal Preparation**—The kinetics of glucose metabolism were studied in chronically cannulated, conscious mice. Cannulas were surgically implanted 4–5 days prior to experimentation. Mice were anesthetized with sodium pentobarbital (70 μg/g body weight, intraperitoneal). Cannulas were surgically implanted into the right jugular vein and the left carotid artery, externalized in the interscapulum and sealed (details available on request). The arterial cannula was used for sampling whole blood from which arterial glucose and metabolite measurements were made. The venous cannula was used to infuse tracer and glucose. Body weight, hematocrit, general appearance, and intra-experimental physiological responses were used as indices of health.

**Experimental Procedures**—Control and heterozygous transgenic (3 GK gene copies) mice were fasted for 6 h prior to experimentation. The experimental protocol was 220 min in duration and consisted of a 100-min equilibration period (0–100 min) followed by a 120-min experimental period (0–120 min). A 2-pCi bolus of tracer (3-3H[glucose], NEN Life Science Products) was given at −100 min, followed by a constant infusion at 0.04 μCi/min for the duration of the sample. Samples for tracer and insulin determinations were taken every 20 min starting at −30 min. The blood glucose concentration was measured at −30, 0, 30, and every 15 min thereafter using a Beckman Glucose Analyzer 2. The kinetics of glucose metabolism were studied using radioisotopic tracer methods in both control animals and transgenic mice with a single extra copy of the GK gene locus. The results of studies performed under basal (6-h fasted) conditions are shown in Table I (A–C), while the glucose turnover rate (Rg) was similar to the two groups (Table I, Fig. 1B, Table I). From these values, a significantly higher glucose clearance rate was calculated for the transgenic mice than for the controls (Fig. 1C, Table I). However, plasma insulin levels (Table I), glycogenolytic rates (Table I), and NEFA (not shown) were not significantly different between the groups. Given the lack of evidence for increased insulin secretion under these conditions, the liver appears responsible for increased glucose clearance rates, consistent with observed transgene expression patterns (1).

**RESULTS AND DISCUSSION**

**Hypoglycemia and Improved Glucose Tolerance in GK Transgenic Mice**—To determine the effect of increased GK gene copy number on blood glucose homeostasis transgenic mice bearing one and two extra copies of the entire GK gene locus were studied. Fed control mice had a plasma glucose concentration of 145 ± 10 mg/dl. Transgenic mice with a single copy of the GK gene locus transgene (heterozygotes) had a plasma glucose concentration that was decreased by 25% (to 109 ± 4 mg/dl; p < 0.05) and mice with two transgene copies (homozygotes) had a plasma glucose concentration that was 37% lower (91 ± 6 mg/dl; p < 0.01). Twenty min after a glucose bolus (1.5 mg/g body weight) the plasma glucose concentrations were 233 ± 21 mg/dl, 172 ± 9 (p < 0.05), and 132 ± 7 mg/dl (p < 0.01) in the controls, heterozygotes, and homozygotes, respectively. These results revealed that animals with increased GK gene copy number have both a reduction in the base-line plasma glucose concentration and an improvement in glucose tolerance.

**Hypoglycemia in Glucokinase Gene Locus Transgenic Mice**—To magnify the metabolic differences caused by the transgene, glucose turnover rates were also assessed under hyperglycemic clamp conditions. The results of these studies are shown in Fig. 1 (D–F), and all pertinent metabolic parameters are summarized in Table II. A variable infusion of 50% glucose was used to maintain blood glucose levels at −300 mg/dl. Over the course of the
experiment, NEFA levels fell while plasma lactate levels rose (Table II), indicating that the animals were healthy and responding appropriately. The average glucose level during these experiments was slightly higher in the transgenic group (Fig. 1D, Table II) as was the glucose turnover rate (Fig. 1E, Table II). After correction for the differences in the clamped glucose concentration, the glucose clearance rates did not differ between the two groups of mice (Fig. 1F, Table II), indicating that both groups of mice metabolized glucose in a similar manner. However, the transgenic animals secreted significantly less insulin in response to the glucose challenge compared with the controls (Table II). This insulin secretory defect is consistent with the observed reduction in islet GK content in these transgenic mice (1). The basis for the insulin secretory defect in the GK transgenic mouse islets is discussed further below.

Since the glucose turnover rates during the hyperglycemic clamp were similar in both groups of mice, despite very different insulin levels, the GK transgenic animals were either globally more sensitive to insulin, or a tissue-specific effect of the
transgene was responsible. As a marker of tissue-specific glucose metabolism, both hepatic and skeletal muscle glycogen content was determined before and after glucose clamp using tissue samples from separate but matched groups of animals. Under hyperglycemic and hyperinsulinemic conditions, the majority of glucose metabolized by liver or muscle is used to synthesize glycogen (6, 40). Prior to the hyperglycemic clamp, there was a similar amount of glycogen in both liver and skeletal muscle between transgenic and control mice (control liver 25 ± 2, transgenic liver 28 ± 3, control skeletal muscle 1.6 ± 0.1, and transgenic skeletal muscle 1.5 ± 0.1 mg of glucose/g of tissue). However, at the end of the 120 min of glucose infusion, the transgenic mice had both more liver glycogen (control 28 ± 2, transgenic 38 ± 1 mg of glucose/g; p < 0.01) and less muscle glycogen (control 4.3 ± 0.6, transgenic 3.1 ± 0.5 mg of glucose/g) than the control animals. Interestingly, the calculated total body glycogen content at the end of the experiment, based on estimated liver and muscle masses, were similar (control 77.6, transgenic 75.8 mg of glucose/mouse).

Downstream Effects of Increased Hepatic GK Gene Expression—To determine whether differences in GK gene expression have the potential to directly affect hepatic glucose metabolism, the effects of the hyperglycemic clamp on L-PK and PEPCK mRNAs were also determined. L-PK and PEPCK determine flux through the glycolytic and gluconeogenic pathways, respectively, in the liver. As shown in Fig. 2, L-PK mRNA was induced by hyperglycemic clamp to a greater degree in transgenic animals compared with controls, while PEPCK mRNA was inhibited to a greater degree in transgenics. Because L-PK gene expression is regulated by glucose metabolism, these results are consistent with increased glycolytic flux in transgenic hepatocytes (28–30). Additionally, the enhanced inhibition of PEPCK gene expression observed during the hyperglycemic suggests that hepatic glucose production is downregulated, in part, as a result of increased glycolytic flux, perhaps through a carbohydrate response element in the PEPCK gene (41). Thus, changes in GK gene copy number can cause alterations in the expression patterns of key hepatic genes that contain metabolic response elements, thereby probably altering flux through the metabolic pathways that these two enzymes control.

Interpretation of the Phenotype—The studies shown above provide new insights into the relationship between GK gene copy number and glucose homeostasis. First, they clearly establish the reciprocal relationship between GK gene copy number and the blood glucose concentration, and demonstrate the high control strength that GK activity exerts on blood glucose homeostasis. Second, they help to clarify the role of the liver in the pathogenesis of MODY-2. Third, they provide new insights into the interactions that occur between the liver and islet, involving the differential regulation of GK, that become clear only through such studies as these. These insights will be discussed individually.

GK Gene Copy Number and Blood Glucose Concentration—The functional loss of one GK gene copy in both GK knock-out mice (6–8) and MODY-2 patients (9, 42) causes an elevation in the blood glucose concentration. Here, by studying transgenic animals that contain extra copies of the entire GK gene locus, we have observed a stepwise reduction in the blood glucose concentration with increasing number of gene copies. Combined, these studies clearly establish the reciprocal relationship between GK gene copy number, and the blood glucose concentration. Furthermore, these studies also reveal the high control strength that GK has on the regulation of whole-body glucose metabolism. A 50% reduction in GK gene copy number (to 1 copy) raises the blood glucose concentration by ~50% (6–8), whereas a 50% increase (to 3 copies), or a 100% increase (to 4 copies), lowers the blood glucose by ~25% and ~37%, respectively. Clearly, these results indicate that small alterations in GK gene expression, both more and less, have the potential to be physiologically significant, consistent with the essential role of this hexokinase in both hepatic and islet glucose metabolism.

Role of the Liver—The hypoglycemic phenotype of the GK transgenic mice appears to be due primarily to increased hepatic GK gene expression (1). Increased GK gene copy number leads to an increase in the efficiency of glucose utilization by the liver. These results are most easily appreciated when modeled on the classical GK glucose dependence curve (see Fig. 3) (43). Two GK activity curves are illustrated, one each for control and transgenic mice, following the equation $V = S^{\max}V_{\max}/S + K_s$, where $K_s = K_{\text{cat}}K_m$ and arbitrarily using a Hill coefficient of 1.65 and a $K_{\text{cat}}$ value of 180 mg/dl (43). Increased hepatic GK gene expression, which results in a relative increase in GK activity per unit of liver (1), causes a leftward shift of the glucose kinetic curve (Fig. 3, dashed line). Under basal conditions, the transgenic mouse liver phosphorylates glucose more efficiently (as shown on the left of Fig. 3). Thus, a similar rate of glucose metabolism is achieved in the transgenic mice, but at a lower plasma glucose concentration, due to the leftward shift of this curve.

The increased glucose metabolism in these mice can also be
interpreted in terms of the GK-Glc-6-Pase hepatic substrate cycle, with the difference between the two activities determining net hepatic glucose flux (19, 44). By increasing GK activity, the forward reaction is favored, the plasma glucose concentration falls, and a new equilibrium point established. However, at steady state, which occurs at a glucose concentration \(\sim 20\) mg/dl lower in mice with three GK gene copies than controls, net flux through this cycle does not differ, as is evident from the similar glucose turnover and glycogenolytic rates, despite the differences in substrate concentration. Thus, increased hepatic GK alters hepatic glucose metabolism by shifting the balance between GK and Glc-6-Pase toward increased glucose phosphorylation, thereby lowering the blood glucose level. In the hyperglycemic clamp experiments, by clamping the substrate concentrations to the same level (shown on the right of Fig. 3), increased hepatic GK activity leads to increased glucose utilization, as is evident by the larger increment in hepatic glycogen content at the end of the experiment.

The elevation in blood glucose concentration that occurs in MODY-2 as a consequence of a reduced number of functional GK gene copies is thought to be due largely to a defect in glucose-stimulated insulin secretion as a result of a reduction of islet GK activity (6–8, 10, 45). However, in the same way that increased GK gene copy number increases hepatic glucose metabolism, a reduction in GK gene expression would be expected to impair liver glucose metabolism. Thus, the elevated blood glucose concentration in MODY-2 is likely to reflect both a new steady state in hepatic glucose flux, as well as a defect in glucose-stimulated insulin secretion. Indeed, it may be the dual defect induced by GK gene mutations (both islet glucose sensing and hepatic glucose utilization) that prevent normal compensatory mechanisms from correcting the blood glucose concentration back toward normal. This issue has also been experimentally addressed by Bahl et al. (6), who used metabolic tracer techniques to study mice heterozygous null for GK and observed a marked decrease in the whole body glucose turnover rate under hyperglycemic clamp conditions.

**Interactions between the Liver and Islet—**An unsuspected finding of these studies is the diminished insulin-secretory response during the hyperglycemic clamp. Increased GK gene copy number was expected to increase both islet and hepatic glucose metabolism. However, the primary effect of increased hepatic glucose metabolism appears to have a secondary effect of attenuating islet GK content, as a consequence of the lower blood glucose concentration. Indeed, the improved ability of the liver to dispose of glucose is nearly perfectly counterbalanced by an insulin secretory defect, resulting in whole body metabolic rates that are not different under either the basal or hyperglycemic clamp conditions, thereby providing further evidence that the net glucose lowering effects of GK in the islet and liver are tightly associated.

Previous studies have shown that glucose is a major regulator of islet GK (25, 26, 46, 47). Indeed, Bedoya et al. (48) were first to show this effect by implanting insulinoma tumors into rats, which by inducing hypoglycemia led to a reduction in islet GK activity. The fact that there is diminished expression of islet GK in these animals, despite the increased GK gene copy number, indicates that the islet adapts to variations in the prevailing blood glucose concentration, thereby leading to reduced insulin secretion. This adaptive response of the islet may be a means of protection against the hypoglycemic effects of too much GK. Interestingly, the liver may also be capable of attenuating the physiological effects of GK overexpression. Increased nuclear localization of GK, presumably reflecting increased binding to GKRp, was observed in the livers of these mice (1). Since binding of GK to GKRp inactivates the enzyme (23, 49), this interaction may help explain why the increment of the change in blood glucose concentration between 2 and 3 gene copies was larger than that between 3 and 4 gene copies. Interestingly, Agius et al. have shown recently that GK binding to GKRp is a saturable process (14). While this suggests that the binding capacity of GKRp can be exceeded at some point, such high levels of GK gene expression were probably not achieved in these mice. Finally, alterations in GK gene expression, as a result of the changes in gene copy number, probably affects other hepatic metabolic pathways, as suggested by alterations in both L-PK and PEPCK mRNA levels in these mice. Thus, mechanisms appear to exist in both the islet and liver that are capable of attenuating the effect of increased GK activity. All of these mechanisms may be important in minimizing deviations from the normal plasma glucose concentration.

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