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Differential expressed genes of change in glucose metabolism in the liver of GCK knockout mice

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Abstract

To investigate the role of liver-specific expression of GCK in the pathogenesis of hyperglycemia and identify candidate genes involved in the mechanisms for onset and progression of MODY2, we examined the differential expression of genes in the liver of liver-specific glucokinase (GCK) knockout mice ($gck^{w/-}$) model at 2 and 26 weeks by suppression subtractive hybridization (SSH). The expression levels of the ACAT2 and PEPCK genes identified by SSH, glycogen Synthase (GS) and glycogen phosphorylase (GP) genes were further examined at different ages by Real time PCR. In addition, we also characterized the expression of mouse GCK mRNA at different ages as well as the fasting blood glucose, serum insulin, GCK activity, total cholesterol (TC), triglyceride (TG) and glycogen content. The results show that, except for 2-week-old $gck^{w/-}$ mice, the fasting blood glucose levels are significantly higher for $gck^{w/-}$ mice ($P < 0.01$) and the GCK activity of $gck^{w/-}$ mice lower about 50% than for GCK wild type ($gck^{w/w}$) mice ($P < 0.05$). The glycogen content of 4-week old and 40-week old $gck^{w/-}$ mice was lower than that of 4-week old and 40-week old $gck^{w/w}$ mice. The GP mRNA levels decrease at 40-week old $gck^{w/-}$ mice compared to age-matched $gck^{w/w}$ mice. PEPCK mRNA decreases at 2-week old $gck^{w/-}$ mice, but increases at 4-week old $gck^{w/-}$ mice ($P < 0.05$). Changes in the expression of PEPCK genes, delayed development of GCK and impaired hepatic glycogen synthesis in liver potentially lead to onset and progression of maturity onset diabetes of the young, type 2 (MODY-2).

Keywords

glucokinase; MODY2; differentially expressed genes

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Introduction

Glucokinase (GCK) plays an essential role in blood glucose homeostasis. In humans, mutation in the GCK gene cause MODY-2 (1, 2), a disease that is characterized by an early onset and persistent hyperglycemia. The glucokinase enzyme is a member of a family of hexokinases that phosphorylate glucose into glucose-6-phosphate as the first step in glycolysis (3). There are two isoforms of GCK, namely, the β -cell-specific and liver-specific isoforms, each of which is characterized by the presence of an isoform specific first exon (4, 5). In β -cells, GCK plays a critical role in glucose-responsive insulin secretion by functioning as a 'glucose sensor'. The enzymatic kinetics of GCK allows β -cells to change the glucose phosphorylation rate over the physiological range of glucose concentrations (6).

In the liver, GCK is thought to determine the rates of both glucose uptake and glycogen synthesis (7, 8). The role of the liver in the pathogenesis of hyperglycemia that characterizes MODY-2 has not yet been clearly resolved. The characterization of the cell-specific roles of GCK in glucose homeostasis requires an animal model in which the enzyme can be selectively eliminated in specific sites without affecting expression at the other sites. We generated a hepatocyte-specific gene knockout of glucokinase in mice (9) using a Cre-loxP gene targeting strategy to identify tissue-specific alterations in glucose metabolism that contribute to pathogenesis. The liver-specific knockout mouse is an ideal animal model as it also could be applied to the investigation of the liver cell-specific roles of GCK in glucose homeostasis and its role in MODY2. In a previous study, our GCK knockout mice were shown to have increases in their blood glucose levels at 4 weeks and at 6 weeks their fasting blood glucose level were significantly higher than in $gck^{w/w}$ mice and displayed impaired glucose tolerance(9). Here we further characterized this mice by examining fasting blood glucose, serum insulin, TC, TG, GCK activity and GCK mRNA levels in both $gck^{w/-}$ and $gck^{w/w}$ mice out to 26 weeks of age. To investigate the reasons for the increases in glucose from 4 weeks of age and to identify genes that change expression, which may lead to the onset and development of MODY2, we also used SSH to screen for genes that are differentially expressed in the livers of liver-specific $gck^{w/-}$ mice at the ages of 2 and 26 weeks..

Material and Methods

Animals

Liver-specific heterozygous $gck^{w/-}$ mice at 2, 4, 16, 26 and 40 weeks of age were used in this study. Age-matched $gck^{w/w}$ mice were used as control animals. The liver specific $gck^{w/-}$ mice was previously constructed by our lab (9). All animal experiments for the "Guidelines for Animal Experiment" and were approved by the Peking University Health Science Center.

Biochemical analysis

Blood glucose concentrations after an 8h fast were measured from the tails of mice with a Roche blood glucose monitor (Glucotrend 2). Serum insulin levels were determined by radioimmunoassay (RIA) using a human insulin RIA kit. Triglyceride (Enzyme method),

and total cholesterol (Enzyme method) were determined using commercial kits (CHANGCHANG, China) and an automatic analyzer (AMS-18C).

Glucokinase activity

GCK activity in the liver was measured using methods that have been previously described (10). Livers were isolated and homogenized using a homogenizer in 3 volumes of ice cold 20 mM KH₂PO₄-KOH (pH 7.0) containing 100 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5% (v/v) glycerol, 1 mg/ml BSA and 1 mM DTT. The homogenates were centrifuged at 160 × g for 30 min. The supernatants were ultracentrifuged for 60 min at 100,000 × g. The supernatants were incubated for 30 min at 37°C with 50 mM HEPES-NaOH, 100mM KCl, 8 mM MgCl₂, 0.5 mM NAD, 1 mM DTT, 1 U/mg G6PDH, 5 mM ATP and 50 mM or 0.5 mM glucose. Glucokinase activity was corrected by subtracting the activity measured at 0.5 mM glucose from the activity measured at 50 mM glucose. Protein concentrations were determined with the Bio-Rad protein assay reagent (11).

Glycogen content

Glycogen content was determined by Kit (NanJingJiancheng Company, China) as the manual.

SSH

SSH were performed with RNA isolated from the livers of 2-week and 26-week old *gck^{w/-}* mice in both directions in order to identify both up-regulated and down-regulated genes. To find overexpressed genes in 26-week-old *gck^{w/-}* mice they were used as the “tester,” while the other 2 week-old *gck^{w/-}* mice were the “driver.” For reverse subtraction, the “tester” and “driver” mice were switched. Total RNA was extracted with TRIZOL (Invitrogen). PolyA⁺ mRNA was isolated using the Oligotex mRNA Midi Kit (Qiagen) and converted to cDNA using a cDNA synthesis kit (Takara). The tester and driver cDNAs were digested with *Rsa*I (Promega). The tester cDNA pool was divided into two portions, each of which was ligated to a different cDNA adapter sequence and two sequential hybridizations were then performed. For the first hybridization, an excess of driver was added to each of the tester pools, the samples were heat denatured and allowed to anneal to each other, resulting in the generation of several different hybrid sequences of cDNA.

For the second hybridization, the two different tester pools are mixed together in the presence of an excess of denatured driver, and new hybrids are formed which consist of differentially expressed cDNAs with different adaptors on each end. The ends of the differentially expressed cDNA sequences are then filled in by DNA polymerase and two rounds of PCR are performed to enrich for these cDNA clones. During the PCR amplification, the non-differentially regulated, hybrid clones are either not amplified or do not exhibit exponential amplification, thus, only the differentially regulated genes are enriched by this process. The resultant pool of cDNA clones obtained from the PCR reaction were shotgun ligated into the pMD-18 T vector (Takara) and transformed into JM109 cells and plated onto Luria-Bertani (LB) agar plates containing ampicillin (100 µg/mL). Recombinant colonies were picked from the plates and sequenced by Invitrogen. The

identity of the clone sequences was determined by BLAST searches against the mouse genome.

Quantification of mRNA by real-time quantitative PCR

Total RNA was extracted from liver using Trizol. High-Capacity cDNA Reverse Transcription kits (Applied Biosystems) were used to synthesize cDNA. Quantitative detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 2 differentially expressed genes (ACAT2 and PEPCK genes), GP, GS and GCK were performed with an iQ5 optical system (BIO-RAD) and the Power SYBR screen PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Primers for each gene (Table 1) were synthesized by Takara. PCR amplification was carried out in a total volume 20 μ L, containing 1 μ L cDNA solution, 10 μ L of 2 $\times$ Power SYBR Green PCR Master Mix, 1 μ L each primer at 10 μ M, and 7 μ L of nuclease-free water. GAPDH was quantified, and were used for the normalization of expression values of the other genes. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was more than 20-fold greater than the standard deviation of the baseline fluorescence (12). The

CT method of relative quantification was used to determine the fold change in expression (13). This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples ($CT = CT_{\text{target}} - CT_{\text{GAPDH}}$), and further normalized with the internal control ($CT = CT_{\text{target}} - CT_{\text{GAPDH}}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$) as described (14).

Statistical analysis

The results are shown as means $\pm$ SEM. Differences between control and experimental groups were evaluated by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

Biochemical parameters

Fasting blood glucose levels were measured in liver-specific GCK knockout ($gck^{w/-}$) and wild type ($gck^{w/w}$) mice at the ages of 2, 4, 16, 26 and 40 weeks of age. Except for 2-week-old $gck^{w/-}$ mice, that the fasting blood glucose levels in the $gck^{w/-}$ mice are significantly higher than those in $gck^{w/w}$ mice at each age ($P < 0.01$) (Fig. 1A). Insulin, TC and TG levels of $gck^{w/-}$ mice show no significant differences compared to age-matched $gck^{w/w}$ mice, indicating that insulin secretion by the pancreas of the $gck^{w/-}$ mice was normal (Fig. 1B, C, D).

GCK activity and GCK mRNA level

The GCK enzymatic activity of $gck^{w/-}$ mice was about 50% of that of age-matched $GCK^{w/w}$ mice, except at 2-weeks of age (Fig. 2A). Expression of the GCK mRNA in $gck^{w/w}$ mice was increased starting from 4-weeks and from 16-weeks of age it remained relatively stable (Fig. 2B). Compared to age-matched mice, a significant difference in GCK expression was only observed at 4-week of age (Fig. 2B).

Glycogen content

The glycogen content of $gck^{w/-}$ mice fell to about 50% of the level of age-matched $gck^{w/w}$ mice at 4-weeks and 40-weeks of age (Fig 4C).

Identification of differentially expressed genes by SSH and Confirmation by quantitative real-time PCR

Approximately 20 differentially expressed genes were identified in our SSH screens (Table 2). To confirm the differential expression of the ACAT2 and PEPCK genes detected by our SSH screen, and to examine if there was differential expression of the GP GS genes, we performed quantitative real-time PCR analysis with GAPDH as an endogenous control.

When compared to age-matched $gck^{w/w}$ mice, the level of the PEPCK mRNA in 2-week-old $gck^{w/-}$ mice was decreased, however by 4 weeks the level of PEPCK mRNA was greater than that found in wild-type mice (Fig. 3A). In both wild-type and gck knockout mice, an age-related increase in ACAT2 gene expression was noted, but there was no significant difference in ACAT2 mRNA levels between $gck^{w/-}$ and age-matched $gck^{w/w}$ mice (Fig. 3B). Likewise, the levels of GS mRNA in $gck^{w/-}$ mice were not different from those of age-matched $gck^{w/w}$ mice (Fig. 4A). GP mRNA did show some differences, with the mRNA levels decreased in the 40-week old $gck^{w/-}$ mice compared to age-matched $gck^{w/w}$ mice (Fig. 4B).

Discussion

Glucokinase (GCK) plays an essential role in blood glucose homeostasis. In both humans and in animal models, mutations in the GCK gene cause MODY-2, a disease that is characterized by early onset and persistent hyperglycemia (1, 2). A liver-specific glucokinase knockout mouse has been constructed by our lab as a model for MODY2, using the Cre-loxP gene targeting strategy, and its primary effects have been evaluated (9). The liver-specific glucokinase knockout mouse is an ideal animal model for MODY2, as it can be used to investigate the liver-specific roles of GCK and can be used to screen anti-diabetic drugs. This study focused on the identification of genes that show differential expression in the liver of liver-specific glucokinase knockout mice as they age and to investigate changes associated with the development of disease.

The diabetic state developed in the $gck^{w/-}$ mice by 4 weeks of age based on the fact that fasting blood glucose levels increased at 4 weeks of age, but were normal at 2 weeks of age. In this study, changes in the physiological parameters of $gck^{w/-}$ mice, including an increase in the fasting blood glucose levels, were observed as early as 4 weeks of age (Fig. 1), which is consistent with our previous findings (9). The hyperglycemia associated with GCK mutations is often mild, with fewer than 50% of subjects presenting overt diabetes (15). Our $gck^{w/-}$ mice, in which gck selectively was knocked out in one of two alleles in the liver, yielding about 50% of the level of expression of wild-type mice, does not affect expression in pancreatic β cells (9).

A detailed examination of the genes for enzymes involved in hepatic glucose metabolism demonstrated that the GCK mRNA levels of $gck^{w/-}$ mice were half those of $gck^{w/w}$ mice at

4 weeks of age, but had matched the increase seen in wild-type mice so that from 16 weeks of age the $gck^{w/-}$ mice and $gck^{w/w}$ had similar mRNA levels (Fig. 2B). The reason for this finding is that the 9th and 10th exons of $gck^{w/-}$ mice are knocked out, but the real time PCR primer of GCK is located between 5th and 6th exon. In previous studies (16, 17) it has been shown that the GCK enzyme activity and GCK expression are absent from the livers of rats up to the age of about 16 days when it then begins to gradually rise, attaining a level of about half the adult level at about 24 days and then the adult level at about 30 days. Our study suggests that a GCK mRNA is already present in $gck^{w/w}$ mice at 14 days and attains a level of about half the adult level at about 28 days. In the $gck^{w/-}$ mice, the GCK mRNA level at 14 days was not different from that at 28 days which suggests that the development of GCK expression is delayed in the knockout mice. The reason for suppressed glucokinase development is unclear. In our study, the change in the GCK mRNA levels likely is partly responsible for the mild increase in the fasting glucose in $gck^{w/-}$ mice from 4 weeks in age.

When $gck^{w/-}$ mice were compared to age-matched $gck^{w/w}$ mice, the GCK enzymatic activity fell to about 50% at 4, 16, 26 and 40 weeks, despite having similar mRNA levels at most ages. These findings suggested that a decrease in GCK activity, which plays a key role in the first step of hepatic glucose metabolism, is an early change in the development of MODY2 and is an important reason for increased fasting glucose in $gck^{w/-}$ mice.

The differential genes involved in glucose and lipid metabolism, PEPCK and ACAT2 were further confirmed. Phosphoenolpyruvate carboxykinase (PEPCK), a gene we detected as being upregulated in 26 week old $gck^{w/-}$ mice, is the rate-limiting enzyme of gluconeogenesis and is encoded by PEPCK gene (18). PEPCK thus is a candidate diabetes and obesity gene (18, 19). In this study, Compared to age-matched $gck^{w/w}$ mice, the PEPCK mRNA level in $gck^{w/-}$ mice was increased at 4 weeks of age, but was decreased at 2 weeks, an age which may also preceded onset of diabetes (Fig. 3). No changes in PEPCK mRNA levels were observed at any other age and may explain the long mild fasting hyperglycemia and failure of progression of the disease.

Acyl-CoA:cholesterol acyltransferase (ACAT) was similarly found to be upregulated in $gck^{w/-}$ mice and is an intracellular enzyme present in various eukaryotic cells and catalyzes the formation of cholesterol esters from long-chain fatty acyl coenzyme A and cholesterol (20). The ACAT enzyme participates in various physiological processes such as lipoprotein assembly and dietary cholesterol absorption (21–23), and plays an important role in foam cell development in atherosclerosis (24, 25). Coronary heart disease, though, has a lower prevalence in MODY2 (26). Despite a difference in expression of ACAT between 26 and 2 week old $gck^{w/-}$ mice, no difference in ACAT2 mRNA levels was seen between $gck^{w/-}$ and age-matched $gck^{w/w}$ mice at any age. The similar levels of expression may explain why the levels of TC and TG are similar in the two groups of mice and the low frequency of coronary heart disease.

Differences in GS mRNA levels were not found between $gck^{w/-}$ mice and $gck^{w/w}$ mice, despite the glycogen content of $gck^{w/-}$ mice at 4 and 40 weeks being lower than that of age-matched $gck^{w/w}$ mice. This observation suggests that the liver-specific glucokinase knockout does not affect the level of GS mRNA expression but may suppress GS enzymatic activity.

GP mRNA levels were lower in 40-week old $gck^{w/-}$ mice than in 40-week old $gck^{w/w}$ mice. Glycogen phosphorylase is known to exert low expression or activity in association with an increase in serum glucose, to decrease glycogen stores, and enhance gluconeogenesis (27, 28). GP mRNA thus likely is decreased as a consequence of the elevation of serum glucose levels and leads to a reduction of glycogen stores. Abnormalities in liver glycogen metabolism may play an important role in the onset of hyperglycemia in $gck^{w/-}$ mice starting at 4 weeks of age.

It is widely recognized that mutations in GCK are a major cause of MODY2. Individuals, who are heterozygous for a mutation in the GCK gene exhibit mild fasting or postprandial hyperglycemia (2, 7, 29, 30) (31) accompanied by impaired hepatic glucose uptake and glycogen synthesis, as well as a defect in glucose stimulated insulin secretion (2). Previous studies have mainly investigated changes in the pancreas and liver (32, 33) whereas we have focused on only the liver to investigate the progression of MODY2 pathology. The liver-specific GCK knockout mouse is an animal model where this enzyme was selectively eliminated from a specific site without affecting expression in other sites, thus providing an opportunity to investigate the cell-specific roles of GCK in glucose homeostasis. Our research has shown that the GCK knockout mouse model develops changes in liver GCK activity and GCK mRNA levels and is mildly hyperglycemic, but does not show a significant change in insulin secretion. This study shows that the development of GCK mRNA expression at 4-weeks in $gck^{w/-}$ mice is delayed, PEPCK mRNA levels are decreased in 2-week old $gck^{w/-}$ mice, but increased at 4-weeks, and that glycogen metabolism is abnormal suggesting that the GCK knockout leads to changes in gene expression and metabolism which contribute to the development of MODY2.

These findings suggest that a disorder of hepatic glucose metabolism, beginning with suppressed glucokinase development and activity, increased PEPCK mRNA and abnormalities in liver glycogen metabolism, are involved in the onset and progression of MODY2. Promoting GCK activity and expression, inhibiting PEPCK gene expression, and improve glycogen metabolism may help to delay and control the onset and progression of MODY2. We provide evidence for the involvement of a hepatic glucose metabolic disorder in the onset and progression of MODY2 and investigated the mechanism of the onset of diseases and potential targets for drug development to prevent pathological progression.

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Fig 1.

Fasting blood glucose concentrations (A), serum insulin concentrations (B), TC (C) and TG (D) of *gck*^{w/-} mice and *gck*^{w/w} mice at different ages. *n*=6. Mean±S.E. ***p*<0.01 vs age-matched *gck*^{w/w} mice.

Fig 2.

GCK mRNA levels (A) and GCK activity in the liver (B) of *gck^{w/-}* mice and *gck^{w/w}* mice at different ages. Data represent means $\pm$ S.E. (n=6). * $p < 0.05$ vs. age-matched *gck^{w/w}* mice.

Fig 3.

PEPCK mRNA levels (A) and ACAT2 mRNA levels (B) in the liver of *gck^{w/-}* mice and *gck^{w/w}* mice at different ages, Data represent means $\pm$ S.E. (n=6). * $p < 0.05$ vs. age matched *gck^{w/w}* mice.

Fig 4.

GS mRNA levels (A), GP mRNA levels (B) and glycogen content (C) in the liver of $gck^{w/-}$ mice and $gck^{w/w}$ mice at different ages. Data represent means $\pm$ S.E. (n=6). * $p < 0.05$ vs. age matched $gck^{w/w}$ mice.

Table 1

Primer for Real time PCR

primer		sequence
GCK	upper	5'CAACTGGACCAAGGGCTTCAA3'
	lower	5'TGTGGCCACCGTGTCATTC3'
PCK1	upper	5'GAACTGAGACTCGCCCTATGTG3'
	lower	5'GTTGCAGGCCCAGTTGTTGA3'
ACAT2	upper	5'AGACTTGGTGCAATGGACTCGAC3'
	lower	5'CATAGGGCCCGATCCAACAG3'
GAPDH	upper	5'TGTGTCCGTCGTGGATCTGA3'
	lower	5'TTGCTGTTGAAGTCGCAGGAG3'
GS	upper	5'ACTGCTTGGGCGTTATCTCTGTG3'
	lower	5'ATGCCCCTCCATGCGTA3'
GP	upper	5'TGGCAGAAGTGGTGAACAATGAC3'
	lower	5'CCGTGGAGATCTGCTCCGATA3'
PCR conditions		50°C × 5 sec, 95°C × 10min, 40 cycles of 92°C × 15 sec and 58°C × 60 sec.

Table 2

Differentially expressed genes identified by SSH

Overexpressed genes	Gene Bank.	Gene
2 weeks	NM_001142706.1	Mus musculus complement factor B (Cfb), transcript variant 2, mRNA
	NM_001161765.1	Mus musculus flavin containing monooxygenase 5, mRNA
	XM_003086541.1	Mus musculus 10-formyltetrahydrofolate dehydrogenase-like, mRNA
	NM_027406.1	Mus musculus aldehyde dehydrogenase 1 family
	NM_153573.1	Mus musculus FK506 binding protein 14 (Fkbp14), mRNA
	NM_010733.2	Mus musculus leucine rich repeat protein 3
	NM_133210.2	Mus musculus SERTA domain containing 3 (Sertad3), mRNA
	NM_008198.2	Mus musculus complement factor B (Cfb), transcript variant1, mRNA
26 weeks	NM_011044.2	Mus musculus phosphoenolpyruvate carboxykinase 1,cytosolic (Pck1), mRNA
	NM_019757.1	Mus musculus fizzy/cell division cycle 20 related 1(Drosophila) (Fzr1), mRNA
	NM_146064.1	Mus musculus sterol Oacyltransferase 2(Soat2), mRNA
	NM_001081067	Mus musculus alpha-1-B glycoprotein (A1bg), mRNA
	NM_011045.2	Mus musculus proliferating cell nuclear antigen (Pcna), mRNA
	NM_011610.3	Mus musculus tumor necrosis factor receptor superfamily, member 1b (Tnfrsf1b), mRNA
	NM_013475.4	Mus musculus apolipoprotein H (ApoH), mRNA
	NM_001136069.2	Mus musculus lactate dehydrogenase A (Ldha), transcript variant 2, mRNA
	NM_001113417.1	Mus musculus thyroid hormone receptor beta (Thrb), transcript variant 1, mRNA