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Roles of hepatic glucokinase in intertissue metabolic communication: Examination of novel liver-specific glucokinase knockout mice

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ABSTRACT

Glucokinase is expressed principally in pancreatic β-cells and hepatocytes, and catalyzes the phosphorylation of glucose to glucose-6-phosphate, a rate-limiting step of glycolysis. To better understand the roles of hepatic glucokinase, we generated *Gck* knockout mice by ablating liver-specific exon 1b. The knockout mice exhibited impaired glucose tolerance, decreased hepatic glycogen content, and reduced *Pklr* and *Fas* gene expression in the liver, indicating that hepatic glucokinase plays important roles in glucose metabolism. It has also been reported that hepatic glucokinase regulates the expression of thermogenesis-related genes in brown adipose tissue (BAT) and insulin secretion in response to glucose. However, the liver-specific *Gck* knockout mice displayed neither altered expression of thermogenesis-related genes in BAT nor impaired insulin secretion by β-cells under a normal chow diet. These results suggest that chronic suppression of hepatic glucokinase has a small influence on intertissue (liver-to-BAT as well as liver-to-β-cell) metabolic communication.

1. Introduction

Glucokinase (GCK, hexokinase type IV) catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P), which is a rate-limiting step in glycolysis [1,2]. Glucokinase is characterized by a high K_m for glucose and a lack of allosteric inhibition by G6P compared to hexokinases I–III. Thus, the rate of glucose phosphorylation is directly proportional to blood glucose levels. Glucokinase is expressed principally in pancreatic β-cells and hepatocytes, but is also present in certain hypothalamic neurons and enteroendocrine cells [2]. The *GCK* gene consists of exons 1a, 1b, and 2–10, and two alternate promoters regulate the tissue-specific expression of exons 1a and 1b [3,4]. Exon 1a is expressed in β-cells, enteroendocrine cells, and neuronal cells, whereas exon 1b is expressed in hepatocytes only. In β-cells, glucokinase serves as a glucose sensor and plays a crucial role in the regulation of insulin secretion [5]. Heterozygous inactivating mutations in the *GCK* gene cause a type of maturity-onset diabetes of the young (MODY2), which is characterized by abnormalities in insulin secretion [4,6]. Homozygous inactivating mutations in the *GCK* gene result in a more severe phenotype presenting at birth as permanent neonatal diabetes [7]. In contrast, activating mutations in the *GCK* gene cause hyperinsulinemic hypoglycemia [8].

Liver glucokinase also plays an essential role in controlling blood glucose levels and maintaining cellular metabolic functions [2]. After glucose is taken up by the liver, it is converted to G6P by glucokinase and stored as glycogen. MODY2 patients reportedly have impaired glucose uptake by liver and decreased accumulation of hepatic glycogen [9,10]. Hepatic glucokinase is also required for the proper activation of glycolytic and lipogenic gene expression in the liver [11]. In addition to these hepatic roles of glucokinase, previous studies have shown that this enzyme is involved in metabolic communication between the liver and different tissues. Adenovirus-mediated overexpression of glucokinase in the liver decreased adaptive thermogenesis by downregulating the expression of thermogenesis-related genes in brown adipose tissue (BAT) [12]. In addition, liver-specific glucokinase knockout mice generated by the Cre/loxP system exhibited impaired insulin secretion in

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response to glucose [13]. These results suggest the presence of intertissue (liver-to-BAT as well as liver-to-β-cell) metabolic pathways.

To understand better the roles of hepatic glucokinase *in vivo*, we generated a new line of *Gck* knockout mice by ablating liver-specific exon 1b. The *Gck* (−/−) mice characterized in the present study exhibited hyperglycemia after glucose load, a defect in hepatic glycogen accumulation, and reduced glycolytic and lipogenic gene expression in the liver. However, these mice displayed neither an insulin secretion defect nor altered expression of thermogenesis-related genes (*Ucp1*, *Pgc1a*, and *Dio2*) in BAT when fed a normal chow diet, suggesting that intertissue regulation by glucokinase is not functional under these conditions. Further studies are necessary to clarify the roles of hepatic glucokinase in intertissue metabolic communication.

2. Materials and methods

2.1. Animals

Construction of the targeting vector and isolation of targeted embryonic stem (ES) cells were carried out using a method described previously [14], except for the use of a 3.86-kb 5′ homologous region upstream and 5.49-kb 3′ homologous region downstream of the ATG codon of exon 1b (Fig. 1A). Chimeric mice were produced by aggregation of ES cells with 8-cell embryos from ICR mice according to a method described previously [14]. Genotyping PCR was performed using a TaKaRa LA PCR Kit with GC buffer (TaKaRa Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The primer sequences were as follows: forward primer (GTP-F1) and reverse primer (GTP-R1) were 5′-AGGAACATCTCTACTTCCCCAACG-3′ and 5′-TGAGCACACTCTGATTGCCCACC-3′ respectively. The PCR product sizes for the wild-type (WT) and knockout mice were 670 bp and 2597 bp, respectively. All mice were kept under specific-pathogen free conditions in a 12-h light/12-h dark cycle with free access to water and normal chow diet (CE-2; CLEA, Tokyo, Japan) in a temperature-controlled room (22 ± 1–2 °C). All experiments were approved by the Kumamoto University Ethics Committee for Animal Experiments.

2.2. Quantitative RT-PCR

Total RNA was extracted from the liver and BAT using Sepasol-RNAI reagent (Nacalai Tesque, Kyoto, Japan). Quantitative RT-PCR was performed using SYBR Premix Ex Taq II (RR820A; TaKaRa) in an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA) as described previously [15]. The relative mRNA level of each gene was normalized to that of 18S rRNA. The specific primers used in this study were as follows. *Gck*: forward 5′-ACAAGGAGGGGAGCCCAGTC-3′, reverse 5′-CCCCCACTTTCACCAGCATC-3′; *Pklr*: forward 5′-GGGGTGACCTTGGCATTGAG-3′, reverse 5′-TTACAGCCTCCACGGGGAAA-3′; *Fas*: forward 5′-TCTGGGCCAACCTCATTGGT-3′, reverse 5′-GAAGCTGGGGGTCCATTGTG-3′; *Hk1*: forward 5′-TGCCTCTGGGCTTCACCTTC-3′, reverse 5′-CCACACAGTCGGTGGCTTTG-3′; *Ucp1*: forward 5′-GGCAACAAGACTGACAGTAAAT-3′, reverse 5′-GGCCCTTGTAAACAAAAAATAC-3′; *Pgc1a*: forward 5′-GGCAACAAGAGCTGAA-3′, reverse 5′-GAATAGGGCTGCGTGCATC-3′; *Dio2*: forward 5′-AGTCAAGAAGGTGGCATTCG-3′, reverse 5′-ACAGCTTCCTCCTAGATGCCT-3′; and *18s rRNA*, forward 5′-GGAGAACTCACTGAGCATGA-3′, reverse 5′-CCAGTGGTCTTGGTGTGCTG-3′.

2.3. Immunoblotting

Tissues were lysed in RIPA buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA, 0.5% sodium deoxycholate, 20 mg/mL Na_3VO_4, 10 mM NaF, 1 mM PMSF, 2 mM DTT, and protease inhibitor cocktail [1/100]) using an ultrasonic homogenizer (BRANSON SONIFIER 250; Branson Ultrasonic Corp, Danbury, CT, USA). Pancreatic islets were isolated by the collagenase digestion method [16]. Hypothalami were collected as described previously [17]. Proteins (liver: 20 μg; pancreatic islets: 15 μg; hypothalamus: 100 μg; small intestine: 100 μg) were separated on a 10% polyacrylamide gel using SDS-PAGE and transferred to a PVDF membrane, which was probed with the primary antibodies. After incubation with the secondary antibodies, proteins were visualized using the Chemi-Lumi One Super Reagent (Nacalai Chemical, Kyoto, Japan) and images were captured using an LAS-1000 analyzer (Fujifilm, Tokyo, Japan). Anti-GCK (H-88; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (AC-15; Sigma-Aldrich, St. Louis, MO, USA) were used as primary antibodies.

2.4. Metabolic studies

For the measurement of G6P, mice were sacrificed at 3 h after feeding following a 24-h fast. Liver tissue (40 mg) was homogenized in perchloric acid by using a Bioruptor (Diagenode, Denville, NJ, USA) and neutralized with 2 M KOH/0.6 M 2-(N-morpholino) ethanesulfonic acid. After centrifugation at 20,000 × *g* at 4 °C for 10 min, the supernatant was subject to a Glucose-6-Phosphate Colorimetric Assay Kit (Biovision Research Products, Mountain View, CA). Hepatic glycogen content was measured as described [18]. Male mice (9–12-week-old) were fasted for 14 h, and glucose (2 g/kg body weight) was injected intraperitoneally. Glucose levels were measured with a glucose sensor (Glutest Neo Super; Sanwa Kagaku, Nagoya, Japan). Serum insulin levels were measured using a mouse insulin ELISA kit (type S) (Shibayagi Co., Gunma, Japan). For the insulin tolerance test (ITT), fourteen-week-old male mice were injected with regular human insulin after a 4-h fast. Total pancreatic insulin content was measured after extraction by the acid-ethanol method [19]. Rectal temperature was monitored using an electronic thermistor (Model BAT-12; Physitemp, Clifton, NJ, USA) equipped with a rectal probe (RET-3; Physitemp, Clifton, NJ, USA) [20].

2.5. Histological analysis

Pancreas was fixed with buffered 10% formalin at room temperature for 3 days. Hematoxylin/eosin (HE) staining was performed using tissue sections (10 μm thick) of paraffin-embedded blocks. To analyze islet mass, sections of paraffin-embedded pancreatic tissues were cut at 100-μm intervals [21]. HE-stained islet images were captured by an all-in-one fluorescent microscope (BZ-9000; KEYENCE, Tokyo, Japan) and the islet areas (μm^2) were measured using ImageJ software.

2.6. Statistical analysis

Data are shown as means ± standard error. The significance of differences was assessed using the unpaired t-test, and $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Generation of novel liver-specific Gck-deficient mice

After electroporation with the targeting vectors, 176 neo-resistant clones were screened for targeted recombination by Southern blot analysis. Using a neo probe, 9 clones gave 9.4- or 11.0-kb bands when digested with *Kpn*I or *Bgl*II, respectively (Fig. 1A). Of

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