Pancreatic β-Cell-specific Targeted Disruption of Glucokinase Gene

DIABETES MELLITUS DUE TO DEFECTIVE INSULIN SECRETION TO GLUCOSE*

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Yasu Terauchi, Hiroshi Sakuratu, Kazuki Yasuda, Keiji Iwamoto, Noriko Takahashi, Kouichi Ito, Haruo Kashiya, Hiroshi Suzuki, Otoya Ueda, Nobuo Kamada, Kouichi Ishage, Kajuro Komedai, Mitsuhiko Noda, Yasunori Kanazawa, Shigeki Taniguchi, Ichitomo Miwa, Yasuo Akanuma, Tatsuhiko Kodama, Yoshio Yazaki, and Takashi Kadowaki

From the Third Department of Internal Medicine, Faculty of Medicine and the First Department of Physiology, Faculty of Medicine, University of Tokyo 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, the Laboratory of Molecular Genetics and Embryology, CSK Research Park, Inc., Komakado, Gotemba 421, the Animal Research Center, Tokyo Medical College, Shinjuku-ku, Tokyo 160, the Omiya Medical Center, Jichi Medical School, Amanuma-cho, Omiya 330, the Laboratory of Pathobiology, Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, and the Institute for Diabetes Care and Research, Ashai Life Foundation, Chiyoda-ku, Tokyo 100, Japan

Mice carrying a null mutation in the glucokinase (GK) gene in pancreatic β-cells, but not in the liver, were generated by disrupting the β-cell-specific exon. Heterozygous mutant mice showed early-onset mild diabetes due to impaired insulin-secretory response to glucose. Homozygotes showed severe diabetes shortly after birth and died within a week. GK-deficient islets isolated from homozygous mice showed defective insulin secretion in response to glucose, while they responded to other secretagogues: almost normally to arginine and to some extent to sulfonylureas. These data provide the first direct proof that GK serves as a glucose sensor molecule for insulin secretion and plays a pivotal role in glucose homeostasis. GK-deficient mice serve as an animal model of the insulin-secretory defect in human non-insulin-dependent diabetes mellitus.

Glucokinase (GK), mainly expressed in pancreatic β-cells and the liver, is thought to constitute a rate-limiting step in glucose metabolism in these tissues (1–4). Since insulin secretion parallels glucose metabolism and the high Km of GK (5–8 mM) ensures that it can change its enzymatic activity within the physiological range of glucose concentrations, GK has been proposed to act as a glucose sensor in the pancreatic β-cell (5, 6). Recently, mutations of the GK gene have been identified in patients with maturity-onset diabetes of the young, a subtype of early-onset non-insulin-dependent diabetes mellitus (NIDDM) (6–8). However, since all the mutations in humans so far occur in the region of the gene that is common to pancreatic β-cells and hepatocytes (9), and are heterozygous, it may not have been possible to fully reveal physiological roles of pancreatic β-cell GK in vitro. To this end, mice carrying a null mutation in the GK gene in pancreatic β-cells, but not in the liver, were generated by homologous recombination. Heterozygous mutant mice showed early-onset mild diabetes resembling the phenotype for human maturity-onset diabetes of the young. Homozygous showed severe diabetes shortly after birth and died within a week. GK-deficient islets showed defective insulin secretion in response to glucose, while they responded to other secretagogues: almost normally to arginine and to some extent to sulfonylureas. These data provide the first direct proof that GK serves as a glucose sensor molecule for insulin secretion and plays a pivotal role in glucose homeostasis.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse GK Gene, Construction of a Targeting Vector, and Homologous Recombinant Experiments—A DNA fragment including the pancreatic β-cell-specific exon 1β of the GK gene was cloned from a BALB/c mouse genomic library (Clontech). A BamHI site was introduced 30 base pairs 3′ to the translation initiation codon of GK by the Kunkel method (10). A neomycin resistance gene (neo) with a pgk-1 promoter but without a poly(A)+ addition signal was substituted for the Xbal-BamHI fragment in the exon 1β. A diphtheria toxin A fragment gene (DTA) with a MC1 promoter was ligated on the 3′ terminus across the vector backbone, for negative selection (11, 12). Homologous recombinant experiments in embryonic stem cells (ES cells) (A3-1) (13, 14) were carried out as described previously (14). These cells were injected into blastocysts from C57BL/6J mice or co-cultured with morulae from C57BL/6J mice (15, 16) and transferred into pseudopregnant ICR females to generate offspring.

Determination of Glucose Phosphorylating Activity—Islets were isolated from the pancreas of 3–7-day-old mice by collagenase digestion method (17). Glucose phosphorylating activities by hexokinase (HK) and GK were determined fluorometrically (18) except that GK activity was measured at 50 mM glucose.

Glucose Tolerance Test—Mice (10 weeks old) were fasted for more than 16 h before the study. They were then loaded with 1.5 mg/g 1H (body weight) glucose by intraperitoneal injection. Blood samples were taken at different time points from the orbital sinus. Insulin levels were determined using an insulin radioimmunossay kit (Shionogi) with rat insulin as standard.

Immunohistochemistry—Pancreata were immersion-fixed in 4.0% (w/v) paraformaldehyde, 0.1 M sodium phosphate buffer at 4°C overnight. Diluted guinea pig anti-porcine insulin (DAKO, A564) (1:200), or rabbit anti-porcine glucagon (DAKO, A565) (1:200), or rabbit anti-human somatostatin (DAKO, A567) (1:200) was applied to the sections for 45 min at room temperature. The sections were then rinsed with Tris-buffered saline, and then treated with a second antibody.

Insulin Content Assay—Isolated islets were suspended in 100 μL of acid ethanol, and cellular insulin was extracted, diluted (100 times), and assayed by radioimmunoassay.

Determination of Intracellular Calcium Concentration—Isolated islets were incubated overnight with RPMI 1640 medium and measure-
**RESULTS AND DISCUSSION**

**Targeted Disruption of Glucokinase Gene in Pancreatic b-cells.**—Alternative splicing of a single GK gene gives rise to two isoforms of GK, one expressed in the pancreatic b-cells and the other in liver, which have different first exons (exon 1b and exon 1L, respectively) (Fig. 1A) (9). These two isoforms are transcribed by two different promoters, and the downstream promoter, which lies between exon 1b and 1L, drives transcription of the liver GK isoform. We were therefore able to disrupt exon 1b expression and thereby selectively eliminate expression of the pancreatic b-cell isoform of GK without affecting expression of the liver isoform. Eight independent ES cell clones were identified as carrying the targeted mutant GK allele (Fig. 1B). Male chimeras originated from two homologous recombinant clones transmitted the mutant GK allele to their offspring. Heterozygous mutant mice were apparently normal and gave birth to mice homozygous for the mutant GK allele. The ratio of wild-type, heterozygous, and homozygous mice was 80:170:85 in 335 offspring at 3–4 days of age, which was consistent with Mendelian inheritance (Fig. 1B). Reverse-transcriptase PCR analysis revealed that GK expression in pancreatic b-islets was completely absent in homozygotes (data not shown). In islets from homozygous neonates, GK activity was completely absent, whereas HK activity was similar to that from wild-type neonates (Fig. 1C). In islets from adult heterozygotes, GK activity (Vmax) was 48% of that from the wild-type mice. In contrast to the b-cell, both GK and HK activities in the liver were unaltered in each genotype (data not shown).

**Mild Diabetes in Heterozygous GK Knock-out Mice.**—At birth, the blood glucose levels of heterozygous and wild-type mice were 2.5 ± 0.3 (n = 5), and 2.4 ± 0.1 (n = 4) mmol liter⁻¹, respectively. However, about 50% of the heterozygous mice showed mild glycosuria within a day, suggesting the development of early-onset diabetes mellitus. Heterozygous mice (10 weeks old) showed significantly higher blood glucose levels both before and after a glucose load and smaller increments in serum insulin levels than wild-type mice (Fig. 2). Insulin tolerance test revealed that the heterozygous mutant mice were as sensitive to insulin as the wild-type (data not shown). These results demonstrate that a heterozygous mutation of the GK gene in the pancreatic b-cells is sufficient to cause impaired insulin secretion to glucose and diabetes mellitus. In this respect, Efrat et al. (21) have generated mice in which pancreatic GK expression was attenuated by a ribozyme-mediated method. Although GK activity of these mice was only 30% that of normal and they showed an impaired insulin response to glucose in perfused pancreas experiments, the fasting and postprandial glucose levels remained normal. It is possible that the different strategies used to attenuate GK expression or the different strains of mice used (Efrat et al. used C3H, whereas this report used ICR) may explain the apparent discrepancy between their and our results.

**Characterization of Homozygous GK Knock-out Mice.**—Ho-
mice of each genotype were fed freely, and blood samples were collected
at 3–4 days of age. The genotype of the neonates was determined by Southern blot analysis (Fig. 1B). The body weight of the
wild-type (open circles), the heterozygotes (open triangles), the homozygotes (crosses) is plotted against the number of days after birth. **, p < 0.01; *, p < 0.05 compared with the wild-type. B, relationship between
the blood glucose levels and the serum insulin levels at 3–4 days of age. Mice of each genotype were fed freely, and blood samples were collected
by decapitation.

Homozygous mutant pups were normal in size, appearance, and
body weight at birth (Fig. 3A). Their blood glucose level was 2.7 ± 0.1 mM (n = 4), which was indistinguishable from that of
wild-type mice (2.4 ± 0.1 mM, n = 4). Although they were able to
suck as evidenced by the presence of milk in their stomachs,
they showed no increase in body weight with age (Fig. 3A). They also showed marked glycosuria within a day, and almost
all animals died within 7 days of birth apparently due to
dehydration. At 3–4 days of age, their blood glucose levels were
markedly higher than those of wild-type or heterozygous ani-
mals, while serum insulin levels were low relative to the ele-
vated insulin contents of the wild-type, heterozygous, and homozygous GK-deficient islets were 1.7 ± 0.3 (n = 5), 2.2 ± 0.7 (n = 6), and 2.5 ± 0.2 (n = 10) ng of insulin/islet, respectively. These
data indicate that β-cell GK is not essential for normal develop-
ment, differentiation of endocrine pancreas, or insulin biosynthesis.

Characterization of GK-deficient Islets—Impact of lack of GK in the pancreatic β-cell was investigated in isolated islets from
7–10 days old mice. A rise in the intracellular Ca2+ concentration in the β-cell ([Ca2+]i) is a key event in glucose-stimulated insulin secretion (19, 20). Although all the GK-deficient islets
looked alike under microscopy, they could be subdivided into two groups in terms of basal [Ca2+]i,levels; those with normal
calcium levels (110 ± 34 nM, n = 7; about 20% of the islets), and
those with higher basal calcium levels (higher than 200 nM;
about 80%) which might reflect some damage of the islets. In
the former group, the increase in [Ca2+]i elicited by glucose was
completely abolished, while the increase evoked by gliben-
clamide or arginine was essentially normal (Fig. 5A). However,
in islets with higher basal calcium levels, the increase in [Ca2+]i elicited not only by glucose but also by glibenclamide was
completely abolished and that by arginine modestly im-
paired (data not shown). The observed heterogeneity of GK-
deficient islets may be due to a direct effect of the impaired
insulin secretion evoked by glucose, but also by glibenclamide
was completely abolished and that by arginine modestly im-
paired (data not shown). The observed heterogeneity of GK-
deficient islets may be due to a direct effect of the impaired
metabolic defects. Even in wild-type or heterozygous GK-deficient islets, there were
islets with higher basal calcium levels (more than 200 nM), but

Fig. 3. Characterization of homozygous GK knock-out mice. A, changes in body weight of neonates. The genotype of the neonates was
determined by Southern blot analysis (Fig. 1B). The body weight of the
wild-type (open circles), the heterozygotes (open triangles), the homozygotes (crosses) is plotted against the number of days after birth. **, p < 0.01; *, p < 0.05 compared with the wild-type. B, relationship between
the blood glucose levels and the serum insulin levels at 3–4 days of age. Mice of each genotype were fed freely, and blood samples were collected
by decapitation.
its proportion was less than 10%.

We next examined insulin secretion using the batch incubation method (Fig. 5B). Insulin secretion from heterozygous GK-deficient islets in response to 0.1 mM or 3 mM glucose was normal, but that in response to 10 mM glucose was significantly impaired compared with wild-type islets. Impairment in insulin secretion in response to 10 mM glucose was less evident. On the other hand, insulin secretion in response to glibenclamide or arginine was unaffected. In homozygous GK-deficient islets, although there was some basal insulin secretion at 0.1 mM glucose, presumably due to the activity of HK, increase in insulin secretion in response to 20 mM glucose was completely abolished. In contrast, insulin secretion in response to arginine was essentially preserved. Regarding insulin secretion in response to glibenclamide, it was decreased by about 50–80% depending on the method of estimation (Fig. 5B). Nevertheless, since the islets used here were supposed to be a mixture of those with normal and those with higher basal calcium levels (expected to be 20% and 80% of the population, respectively), which were responsive and unresponsive to glibenclamide in calcium study, we interpreted these results as suggesting that islets with normal basal calcium levels may have responded to sulfonylurea in insulin secretion.

The insulin secretory response to a physiological increment in glucose concentration was impaired in heterozygous GK-deficient islets and completely defective in homozygous GK-deficient islets despite the presence of HK, supporting the concept that GK serves as a glucose sensor molecule for insulin secretion. This is consistent with the smaller increments in serum insulin levels after a glucose load in heterozygous mice (Fig. 2), lack of increments in insulin levels in homozygous mice in spite of hyperglycemia (Fig. 3B), and the secretory abnormalities in human subjects with GK mutations (23). GK-deficient islets responded to non-glucose secretagogues in insulin secretion (almost normally to arginine and to some extent to sulfonylureas), indicating that GK is not absolutely required for insulin secretion in response to these secretagogues. It should also be noted that insulin secretion in response to glibenclamide was impaired, suggesting that GK may play an important role in insulin secretion in response to some non-glucose secretagogues such as glibenclamide. This possibility would be examined in future. The heterozygous or insulin-treated homozygous mutant mice described here provide the first animal model of diabetes with a defined genetic defect in insulin secretion, and should give important insights into the pathogenesis and development of human NIDDM.

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REFERENCES

1. Matschinsky, F. M. (1990) Diabetes 39, 647–652
2. Magnuson, M. (1990) Diabetes 39, 523–527
3. Matschinsky, F., Liang, Y., Kesavan, P., Wang, L., Froguet, P., Velho, G., Cohen, D., Permutt, M. A., Tanizawa, Y., Jetton, T. L., Niswender, K., and Magnuson, M. A. (1993) J. Clin. Invest. 92, 2092–2098
4. Pilitsis, S. J., Weber, I. T., Harrison, R. W., and Bel, G. I. (1994) J. Biol. Chem. 269, 21925–21928
5. Randle, P. J. (1993) Diabetologia 36, 269–275
6. Vionnet, N., Stoffel, M., Takeda, J., Yasuda, K., Bell, G. I., Zouali, H., Lesage, S., Velho, G., Iris, F., Passa, Ph., Froguet, Ph., and Cohen, D. (1992) Nature 356, 721–722
7. Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Anai, M., Miyazaki, J., Tsukuda, K., Ikuchih, M., Yazaki, Y., and Oka, Y. (1992) Lancet 340, 1316–1317
8. Sakurai, Y., Etoh, K., Kadokawa, H., Shimokawa, K., Ueno, H., Koda, N., Fukushima, Y., Akiyama, Y., Yazaki, Y., and Kadokawa, T. (1992) J. Clin. Endocrinol. Metab. 75, 1571–1573
9. Magnuson, M. A., and Shelton, K. D. (1989) J. Biol. Chem. 264, 15936–15942
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Yagi, T., Aizawa, S., Tokunaga, T., Shigetani, Y., Takeda, N., and Ikawa, Y. (1993) Nature 366, 742–745
12. Tamemoto, H., Kadokawa, T., Tobe, K., Yagi, T., Sakurai, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kabayag, Y., Satoh, S., Sekihara, H., Yoshiko, S., Horiuchi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y., and Aizawa, S. (1994) Nature 372, 182–185
13. Azuma, S., and Toyoda, Y. (1991) Jpn. J. Anim. Reprod. 37, 37–43
14. Kuriloh, Y., Kuriloh, H., Suzuku, H., Komana, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., Kamada, N., Ishihara, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, N., and Yazaki, Y. (1994) Nature 372, 182–185
15. Suzuki, H., Kamada, N., Ueda, O., Ishihara, K., Kuriloh, H., Kuriloh, Y., Komana, T., Yazaki, Y., Azuma, S., and Toyoda, Y. (1994) J. Biol. Chem. 269, 260, 3340–3345
16. Weinhaus, A. J., Poronnik, P., Cook, D. I., and Tuch, B. E. (1995) Diabetes 44, 118–124
17. Graf, S., Leiser, M., Wuy, Y., Fusco-DeManno, D., Emran, O., Surana, M., Jetton, T. L., Magnuson, M. A., Weir, G., and Fleisher, N. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4582–4585
18. Suzuki, H., Komana, N., Ueda, O., Ishihara, K., Kuriloh, H., Kuriloh, Y., Komana, T., Yazaki, Y., Azuma, S., and Toyoda, Y. (1994) J. Biol. Chem. 269, 40, 361–365
19. Gotoh, M., Maki, K., Iyoi, Y., Shimura, Y., and Miwa, Y. (1985) Transplantation 40, 437–438
20. Miwa, I., Mita, Y., Murata, T., Okuda, J., Sugimura, M., Yamada, Y., and Chiba, T. (1995) Enzyme Protein, in press
21. Gryniewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3420–3425
22. Weinhaus, A. J., Poronnik, P., Cook, D. I., and Tuch, B. E. (1995) Diabetes 44, 118–124
23. Byrne, M. M., Sturis, J., Clermont, K., Vionnet, N., Pueyo, M. E., Stoffel, M., Takeda, J., Passa, P., Cohen, D., Bel, G. I., Velho, G., Froguet, P., and Polonsky, K. S. (1994) J. Clin. Invest. 93, 1120–1130
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