Glucokinase and IRS-2 are required for compensatory β cell hyperplasia in response to high-fat diet–induced insulin resistance

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Glucokinase (Gck) functions as a glucose sensor for insulin secretion, and in mice fed standard chow, haplo-insufficiency of β cell–specific Gck (Gck+/−) causes impaired insulin secretion to glucose, although the animals have a normal β cell mass. When fed a high-fat (HF) diet, wild-type mice showed marked β cell hyperplasia, whereas Gck−/− mice demonstrated decreased β cell replication and insufficient β cell hyperplasia despite showing a similar degree of insulin resistance. DNA chip analysis revealed decreased insulin receptor substrate 2 (Irs2) expression in HF diet–fed Gck+/− mouse islets compared with wild-type islets. Western blot analyses confirmed upregulated Irs2 expression in the islets of HF diet–fed wild-type mice compared with those fed standard chow and reduced expression in HF diet–fed Gck+/− mice compared with those of HF diet–fed wild-type mice. HF diet–fed Irs2−/− mice failed to show a sufficient increase in β cell mass, and overexpression of Irs2 in β cells of HF diet–fed Gck+/− mice partially prevented diabetes by increasing β cell mass. These results suggest that Gck and Irs2 are critical requirements for β cell hyperplasia to occur in response to HF diet–induced insulin resistance.

Introduction

Glucokinase (Gck) catalyzes the conversion of glucose into glucose-6-phosphate, and more than 30 years ago Matschinsky and Ellerman proposed that it is critical for glucose sensing (1). Spon-
taneous inactivating mutations of the Gck gene coupled with autosomal-dominant inheritance patterns have been identified in maturity-onset diabetes of the young (MODY) patients (2). These mutations are found in the regions common to the β cell–specific isofrom and liver isofrom, and both decreased β cell function and impaired glucose uptake by the liver have been suggested to be involved in the pathogenesis of MODY (2). We have previously shown that haploinsufficiency of β cell–specific Gck (Gck−/+) leads to mild diabetes associated with impaired insulin secretion in response to glucose (3). Gck is now recognized as functioning as a glucose sensor for insulin secretion by pancreatic β cells (4).

Human type 2 diabetes is characterized by 2 major features: peripheral insulin resistance and impaired insulin secretion by pancreatic β cells (5, 6). Blood glucose levels are maintained within the normal range by adaptations of β cell mass and/or function as a compensatory response to insulin resistance. It should be noted that only 15%–20% of obese or severely insulin-resistant subjects become diabetic; the others maintain normoglycemia via β cell compensation (7). Diabetes only develops when insulin secretion by β cells is insufficient to compensate for the insulin resistance. For example, while both insulin receptor substrate 1 (IRS-1) knockout (Irs1−/−) mice (8, 9) and IRS-2 knockout (Irs2−/−) mice (10, 11) exhibit similar degrees of insulin resistance, Irs1−/− mice have normal glucose tolerance as a result of compensatory β cell hyperplasia, whereas Irs2−/− mice develop diabetes because of a lack of β cell hyperplasia in response to insulin resistance. The prevalence of diabetes has increased markedly in both Western countries and Japan, and the increase can be explained by drastic changes in lifestyle, such as a high-fat diet and sedentary lifestyle. Hypertro-
phyic adipocytes produce an excess of hormones and nutrients that have been reported to cause insulin resistance, such as FFAs (12), and secrete less adiponectin, which has been reported to increase insulin sensitivity (13). Tumor necrosis factor α is secreted by macrophages residing within hypertrophied adipose tissue and causes insulin resistance (14). Under insulin-resistant conditions, blood glucose levels are maintained within the normal range by adaptations of β cell mass (hyperplasia) (8, 9). Glucose itself (7); insulin (15); IGFs (16); transcription factors such as insulin promoter factor 1 (Ipf1) and FoxO1 (refs. 17, 18); tyrosine kinase pathways including the insulin receptor (Insr) (19), IGF1 receptor (Igf1r) (19), Irs2 (10, 11), and Akt (20, 21) pathways; the prolactin signaling pathway (22); and Hgf (23) have been reported to be implicated in β cell growth; however, coordinated regulation of β cell mass by these factors under high-fat (HF) diet–induced insulin-resistant conditions has not been fully elucidated. To establish an animal
model representative of human type 2 diabetes, we fed wild-type mice and Gck<sup>+/–</sup> mice a HF diet and investigated their glucose homeostasis and β cell mass and function. On the HF diet, wild-type mice showed marked β cell hyperplasia, whereas Gck<sup>+/–</sup> mice demonstrated insufficient β cell hyperplasia despite the presence of a similar degree of insulin resistance. Additionally, expression of Irs2 was upregulated in the islets of wild-type mice on the HF diet but markedly lower in those of HF diet–fed Gck<sup>+/–</sup> mice, and overexpression of Irs2 in β cells of HF diet–fed Gck<sup>+/–</sup> mice partially prevented diabetes by increasing β cell mass. Thus, Gck and Irs2 are critical requirements for β cell hyperplasia to occur in response to HF diet–induced insulin resistance.

Results

Early development of diabetes in Gck<sup>+/–</sup> mice on a HF diet. When we fed 8-week-old male wild-type and Gck<sup>+/–</sup> mice a HF diet or standard chow, the 2 genotypes showed similar amounts of body weight gain, adipocyte size, and serum FFA levels that were significantly greater on the HF diet than on standard chow (Figure 1, A–D). After 20 weeks, the glucose-lowering effect of insulin was markedly impaired in both groups on the HF diet compared with those fed standard chow (Figure 1E). After 4 weeks on the HF diet, the wild-type mice maintained normal glucose tolerance as a result of compensatory hyperinsulinemia, whereas the Gck<sup>+/–</sup> mice developed severe diabetes because of a lack of compensatory hyperinsuline-
linemia (Figure 1, F and G, left panels), despite the similar degrees of obesity and insulin resistance shown by both HF diet–fed groups. Although the wild-type mice on the HF diet developed mild dia-
"beta"tes by 20 weeks, because of the compensatory hyperinsulinemia they never developed severe diabetes (Figure 1, F and G, right panels). After 20 weeks, the insulin-to-glucose ratio 30 minutes after a glucose load (the insulin secretory index) revealed a signifi-
cantly increased insulin response to glucose in the wild-type mice on the HF diet compared with those fed standard chow, whereas the \textit{Gck}^{+/−} mice on the HF diet had an insulin secretory index similar to that of the \textit{Gck}^{+/−} mice on standard chow (Figure 1H), indicating that no compensatory increase in insulin secretion occurred in the \textit{Gck}^{+/−} mice while on the HF diet.

\textbf{Figure 2}
Failure of compensatory \(\beta\) cell hyperplasia in HF diet–fed \textit{Gck}^{+/−} mice caused by decreased \(\beta\) cell replication rate. (A) Histologic analysis of pancreatic islets of wild-type and \textit{Gck}^{+/−} mice after 20 weeks on standard chow or HF diet. Sections were double stained with anti-insulin antibody and a cocktail of anti-glucagon, anti-somatostatin, and anti-pancreatic polypeptide antibodies. Representative islets are shown. Red stain, \(\beta\) cells; brown stain, non–\(\beta\) cells. Scale bars: 100 \(\mu\)m. (B) Quantitation of \(\beta\) cell and non–\(\beta\) cell mass in wild-type and \textit{Gck}^{+/−} mice after 20 weeks on standard chow or HF diet. Areas of \(\beta\) or non–\(\beta\) cells (\(\alpha\), \(\delta\), and pancreatic polypeptide cells) are shown relative to total pancreas area (\(n = 4\)). (C) Changes in \(\beta\) cell mass on HF diet. Shown is \(\beta\) cell area relative to pancreas area (\(n = 4\)) after 4, 20, and 40 weeks on HF diet. (D) Number of cells in wild-type and \textit{Gck}^{+/−} mouse islets after 20 weeks on standard chow or HF diet (\(n = 6\)). (E) and (F) Replication rate of \(\beta\) cells, assayed (E) on the basis of BrdU incorporation after 20 weeks on standard chow or HF diet or (F) by PCNA staining after 20 weeks on HF diet. Results are shown as ratios of double-positive cells to insulin-positive cells (\(n = 4\)). Values represent mean ± SEM. *\(P < 0.05\); **\(P < 0.01\).

Failure of compensatory \(\beta\) cell hyperplasia in \textit{Gck}^{+/−} mice on the HF diet. Serum insulin levels are governed by a combination of the insulin secretory capacity of individual \(\beta\) cells and the number of \(\beta\) cells. In this study, we investigated islet mass first. Histologic analysis after 20 weeks on standard chow or HF diet revealed that the HF diet caused islet hyperplasia in the wild-type mice but, surprisingly, no islet hyperplasia in the \textit{Gck}^{+/−} mice (Figure 2A). Quantitative determinations of \(\beta\) cell and non–\(\beta\) cell mass showed that \(\beta\) cell mass increased by 110\% in wild-type mice on the HF diet compared with those on standard chow, but that there was no significant increase in \textit{Gck}^{+/−} mice on the HF diet compared with those on standard chow (Figure 2B). After 4, 20, and 40 weeks on the HF diet, the wild-type mice showed 1.2-fold, 2.0-fold, and 10-fold increases, respectively, in...
β cell mass compared with wild-type mice on standard chow, whereas even after 40 weeks the Gck+/– mice showed only a 2-fold increase in β cell mass (Figure 2C). The number of cells per islet was significantly increased in wild-type mice on the HF diet compared with those on standard chow, but the difference between the 2 Gck+/– groups was not significant (Figure 2D). DNA content per islet was also significantly higher in wild-type mice than in Gck+/– mice on the HF diet (HF diet–fed wild-type, 39.1 ± 2.3 ng/islet, n = 6; HF diet–fed Gck+/–, 22.7 ± 1.9 ng/islet, n = 6; P < 0.001). To estimate the contribution of the size of individual β cells to the increase in β cell mass, we divided the β cell area by the number of β cell nuclei it contained. However, since the results showed little difference in β cell size between wild-type and Gck+/– mice on the HF diet (HF diet–fed wild-type, 155 ± 4 μm², n = 101; HF diet–fed Gck+/–, 149 ± 4 μm², n = 105; P = NS), the increased β cell mass in wild-type mice on the HF diet was attributed to an increase in the number of cells (hyperplasia) rather than to an increase in the volume of individual cells (hypertrophy).

The number of β cells present is governed by a balance among β cell replication (increase in number of β cells by preexisting β cells), neogenesis (generation of β cells by non-β cells, such as acinar cells and duct cells), and apoptosis. We estimated β cell proliferation on the basis of BrdU incorporation and proliferating cell nuclear antigen (PCNA) staining. On the HF diet, there were significantly more insulin and BrdU double-positive cells in the wild-type mice than in the Gck+/– mice (Figure 2E), and similar results were obtained by PCNA staining (Figure 2F). Single-strand DNA analysis revealed no differences in the percentage of apoptotic cells in islets between wild-type and Gck+/– mice on the HF diet (HF diet–fed wild-type, 0.031% ± 0.026%, n = 86; HF diet–fed Gck+/–, 0.018% ± 0.018%, n = 33; P = NS). Fewer than 1 in 3,000 cells in the islets of both mouse groups were found to be apoptotic in an in situ cell death detection kit. Thus, in contrast to the wild-type mice, the failure of compensatory β cell hyperplasia in the Gck+/– mice on the HF diet was associated with a lack of compensatory increase in β cell proliferation.

Impaired glucose-stimulated insulin secretion associated with decreased glucose metabolism in the β cells of Gck+/– mice on the HF diet. Next, we investigated glucose-stimulated insulin secretion (GSIS) by individual β cells. After 4 weeks, GSIS at 22.2 mM glucose normalized by cell number was lower in wild-type mice on the HF diet than in those fed standard chow (Figure 3A). Thus, the hyperinsulinemia in the wild-type mice on the HF diet can be explained by increased β cell mass, not by increased insulin secretion by individual β cells. GSIS at 22.2 mM glucose normalized by cell number was significantly lower in Gck+/– mice than in wild-type mice on both diets, a finding consistent with the results of our previous study (24). The hyperglycemia itself may have also affected the insulin secretory function of islets in the Gck+/– groups. GSIS at 22.2 mM glucose was lower in the islets of the HF diet groups than in those of the standard chow groups of both genotypes, and after 20 weeks it was significantly decreased in the HF diet groups compared with the standard chow groups (data not shown), a finding consistent with previous reports that prolonged exposure to FFA results in suppression of insulin release (25, 26). While islet hexokinase activity was similar in all 4 groups, Gck activity in the Gck+/– standard chow and HF diet groups was 30% and 25% lower than in the wild-type standard chow and HF diet groups, respectively, although the differences were not statistically significant (Figure 3B). When islets of essentially the same size were prepared and [U-14C]glucose oxidation was assayed in their mitochondria, glucose oxidation at 22.2 mM glucose was significantly lower in the HF diet than in standard chow groups of both phenotypes (Figure 3C). Importantly, after 20 weeks on the HF diet, glucose oxidation at 22.2 mM glucose did not significantly differ between wild-type and Gck+/– mice (Figure 3C), suggesting that normal glucose oxidation levels are not necessary for the compensatory increase in β cell mass, although they may be essential for GSIS.

Gene expression profiles of the islets of HF diet–fed mice. We performed a DNA microarray analysis as a means of systematically examining the gene expression profiles of the islets. Of the 12,490 genes examined, 81 were overexpressed (by 3-fold or more; Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI17645DS1) and 63 were underexpressed (by 3-fold or more; Supplemental Table 2) in the islets of Gck+/– mice after 20 weeks on standard chow or HF diet. While islet hexokinase activity was similar in all 4 groups, Gck activity in the Gck+/– standard chow and HF diet groups was 30% and 25% lower than in the wild-type standard chow and HF diet groups, respectively, although the differences were not statistically significant (Figure 3B). When islets of essentially the same size were prepared and [U-14C]glucose oxidation was assayed in their mitochondria, glucose oxidation at 22.2 mM glucose was significantly lower in the HF diet than in standard chow groups of both phenotypes (Figure 3C).}

**Figure 3**

Decreased insulin secretion and glucose oxidation in Gck+/– islets. (A) Static incubation study of islets from wild-type and Gck+/– mice after 4 weeks on standard chow or HF diet. Static incubation of 10 islets/tube was performed at 37°C for 1 hour with various glucose concentrations after preincubation with a 2.8-mM glucose concentration for 20 minutes. Results are shown as pg insulin/cell/h (n = 4). (B) Gck and hexokinase (HK) activity of islets. Glucose phosphorylation activity was assessed in pancreas islets from wild-type and Gck+/– mice after 20 weeks on standard chow or HF diet. Results are shown as mol/kg DNA/h (n = 16–20). (C) Glucose oxidation by pancreas islets from wild-type and Gck+/– mice after 20 weeks on standard chow or HF diet. Results are shown as mol/kg DNA/h (n = 10). *P < 0.05; **P < 0.01.
was slightly, but significantly, lower in the islets of Gck\(^{−/−}\) mice on the HF diet than in those of wild-type mice on the HF diet (Figure 4, B and C). Immunostaining revealed clearly lower Ipf1 nuclear expression in the β cells of Gck\(^{−/−}\) mice on the HF diet than in those of wild-type mice on the HF diet (Figure 4, B and C). In summary, DNA microarray and RNA and protein analyses revealed upregulation of Irs2 and Igf1r expression in the islets of wild-type mice on the HF diet compared with those fed standard chow as well as reduced expression in the islets of Gck\(^{−/−}\) mice on the HF diet compared with those of HF diet–fed wild-type mice.

### Table 1

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### Table 1

Changes in gene expression levels in islets based on DNA microarray analysis. Ratios are based on comparisons of Gck\(^{−/−}\) mice versus wild-type mice after 20 weeks on the HF diet. NC, no significant change.

such as Casp3 and Bad in the islets. RT-PCR analysis confirmed the upregulation of Irs2, Igf1r, and Prtl expression in the islets of wild-type mice on the HF diet compared with those on standard chow as well as reduced expression in the islets of Gck\(^{−/−}\) mice on the HF diet compared with those of HF diet–fed wild-type mice (Figure 4A). Quantitative PCR analysis, which amplified another region of the Irs2 and Igf1r genes, revealed that expression of Irs2 and Igf1r in the islets of Gck\(^{−/−}\) mice on the HF diet was 60.6% (P < 0.01 versus wild-type mice) and 76.4%, respectively, that of their expression level in the islets of wild-type mice on the HF diet.

Next, we examined the protein levels by Western blotting. We prepared islets (less than 250 μm in diameter) from mice after 20 weeks on each diet. The results confirmed upregulation of Irs2 and Igf1r expression in the islets of wild-type mice on the HF diet compared with those on standard chow as well as reduced expression in the islets of Gck\(^{−/−}\) mice on the HF diet compared with those of wild-type mice on the HF diet (Figure 4, B and C). Interestingly, expression of Insr was significantly increased in the HF diet–fed groups compared with standard chow–fed groups (Figure 4, B and C), although there were no differences in expression of Akt1 among the 4 groups. Ipf1 expression was indistinguishable between the islets of Gck\(^{−/−}\) and wild-type mice on standard chow, but the protein level was slightly, but significantly, lower in the islets of Gck\(^{−/−}\) mice on the HF diet than in those of wild-type mice on the HF diet (Figure 4, B and C). Immunostaining revealed clearly lower Ipf1 nuclear expression in the β cells of Gck\(^{−/−}\) mice on the HF diet than in those of wild-type mice on the HF diet (Figure 4, B and C). In summary, DNA microarray and RNA and protein analyses revealed upregulation of Irs2 and Igf1r expression in the islets of wild-type mice on the HF diet compared with those fed standard chow as well as reduced expression in the islets of Gck\(^{−/−}\) mice on the HF diet compared with those of HF diet–fed wild-type mice.

**Insufficient increase in β cell mass in Irs2\(^{−/−}\) mice on the HF diet.** Next we investigated the role of Irs2 in the regulation of β cell mass in mice on the HF diet. After 10 weeks on the HF diet, Irs2\(^{−/−}\) mice exhibited increases in body weight, blood glucose, serum insulin, and insulin resistance similar to those of HF diet–fed wild-type mice (Figure 5, A–D). The Irs2\(^{−/−}\) mice had β cell area and maximum islet diameter similar to those of wild-type mice fed standard chow, but the increases were significantly smaller than those observed in HF diet–fed wild-type mice (Figure 5, E and F). After only 5 weeks on the HF diet, Irs2\(^{−/−}\) mice showed marked aggravation of glucose intolerance (27). While the mean of the maximum islet diameter was 14% greater in wild-type mice on the HF diet than in those on standard chow, a difference that was significant, there was no such increase in the Irs2\(^{−/−}\) mice on the HF diet compared with those on standard chow (data not shown). These results support a role for Irs2 in the increase in β cell mass on the HF diet.

**Transgenic rescue by crossing Gck\(^{−/−}\) mice with β cell Irs2 transgenic mice.** To directly test our hypothesis that reduction of Irs2 explains the impaired β cell hyperplasia in Gck\(^{−/−}\) mice on HF diet, we crossed Gck\(^{−/−}\) mice with β cell Irs2 transgenic (βIrs2Tg) mice, which express Irs2 in β cells under the control of the rat insulin promoter, and generated 2 lines of βIrs2Tg mice (Supplemental Figure 2, A and B). While the β cells of βIrs2Tg6 mice expressed a low level of Irs2 (2.2-fold upregulation compared with wild-type littermates), the β cells of βIrs2Tg12 mice expressed a high level of Irs2 (17-fold upregulation). We chose to use the βIrs2Tg6 line because the 2.2-fold upregulation of Irs2 in βIrs2Tg6 mice was comparable to the Irs2 expression level in wild-type mice on the HF diet. We crossed βIrs2Tg6 mice with Gck\(^{−/−}\) mice and obtained 4 genotypes: wild-type, βIrs2Tg, Gck\(^{−/−}\), and βIrs2TgGck\(^{−/−}\). Only male mice were used in the experiments. After 20 weeks on the HF diet, the fold change in Irs2 protein expression compared with Gck\(^{−/−}\) mice was 3.5 ± 0.2 for wild-type, 4.6 ± 0.5 for βIrs2Tg, 1.0 ± 0.1 for Gck\(^{−/−}\), and 3.1 ± 0.6 for βIrs2TgGck\(^{−/−}\) mice (Supplemental Figure 2, C and D). These results can be explained by the fact that HF diet upregulated endogenous Irs2 expression in the islets of wild-type and βIrs2Tg mice, but not the islets of Gck\(^{−/−}\) or βIrs2TgGck\(^{−/−}\) mice. After 4 weeks on the HF diet, the glucose tolerance of βIrs2Tg mice was similar to that of wild-type mice, but the βIrs2TgGck\(^{−/−}\) mice had better glucose tolerance than did Gck\(^{−/−}\) mice (data not shown). After 20 weeks on the HF diet, the βIrs2Tg mice had glucose tolerance similar to that of wild-type mice, but the βIrs2TgGck\(^{−/−}\) mice had better glucose tolerance and higher serum insulin levels than did Gck\(^{−/−}\) mice (Figure 6, A and B). Thus, overexpression of Irs2 partially prevented diabetes in Gck\(^{−/−}\) mice on the HF diet.

Figure 6C shows representative insulin staining of the pancreas after 20 weeks on the HF diet. Measurement of the β cell area showed that it was significantly increased in βIrs2TgGck\(^{−/−}\) mice compared with Gck\(^{−/−}\) mice (Figure 6D), and consistent with this finding, there were significantly more insulin and BrdU double-positive cells in
βGck+/- mice to Gck+/- mice (Figure 6, C and E). Next we isolated islets from the 4 mouse groups after 20 weeks on the HF diet and carried out static incubation experiments. GSIS at 11.1 mM glucose was not restored in βGck2TgGck-/- mice compared with Gck-/- mice (Figure 6F), indicating that overexpression of Irs2 failed to restore β cell function in Gck-/- mice.

Mechanisms of Irs2 upregulation in the β cells of HF diet-fed mice. We examined known regulators of Irs2 expression, such as cAMP, and subsequent phosphorylation of cAMP response element–binding protein (CREB) (28) and FoxO1 (29). Although the cAMP content of the islets was unaffected by the HF diet (data not shown) and the levels of expression of cAMP-responsive genes — including Creb1, Gip1r, Prkar1a, Prkar1b, Prkar2a, Prkaca, Prkacb, and Lasp1 — were unaltered (Table 1), Ser133 phosphorylation of CREB was impaired in Gck-/- mice compared with wild-type mice (Figure 7, A and B). FoxO1 is an activator of Irs2 in liver, because the Irs2 promoter is activated by FoxO1 through an insulin response element (29), but it has also been characterized as a key distal mediator of insulin signaling, because FoxO1 haploinsufficiency reverses β cell failure in Irs2-/- mice (30). Immunohistochemical analysis revealed that FoxO1 was more localized in the nuclei of β cells of Gck-/- mice on the HF diet than in those of wild-type mice on the HF diet (Figure 8A). In fact, the ratio of nuclear FoxO1-positive cells to the total number of islet cells was significantly higher in Gck-/- mice on the HF diet than in wild-type mice on the HF diet (Figure 8B).

Oversexpression of Irs2 in β cells decreased the number of nuclear FoxO1-positive cells in the Gck-/- mice on the HF diet, indicating that upregulation of Irs2 in β cells stimulated FoxO1 nuclear exclusion (Figure 8, A and B).

The above findings, together with the fact that cAMP and calcium pathways trigger CREB Ser133 phosphorylation, thereby upregulating Irs2 (28), suggest that impaired CREB Ser133 phosphorylation is a plausible explanation for the insufficient upregulation of Irs2 in Gck-/- mice on the HF diet. The significance of the decreased FoxO1 nuclear exclusion in Gck-/- mice on the HF diet are discussed below.

Discussion

Gck is known to function as a glucose sensor in insulin secretion by pancreatic β cells (4), but to our knowledge, its involvement in the regulation of β cell mass had previously not been recognized. In this paper, we report 4 findings, which we believe to be novel, that link Gck to β cell mass. First, we found that wild-type mice on a HF diet showed marked compensatory β cell hyperplasia associated with increased replication of β cells, whereas Gck-/- mice failed to show as much β cell hyperplasia despite showing a similar degree of insulin resistance (Figure 2). The finding that haploinsufficiency of Gck led to insufficient β cell hyperplasia on the HF diet suggests a critical requirement for Gck, not only for GSIS, but for β cell hyperplasia in response to HF diet–induced insulin resistance to protect against diabetes. Although glucose itself has been recognized to be a nutrient for β cells, to our knowledge it was previously unknown whether glucose metabolism is crucial for β cell growth (7). The results of this study in regard to the role of glucose metabolism and Gck in the regulation of β cell mass clearly demonstrate that Gck is associated with β cell mass and proliferation, raising the possibility that other enzymes involved in glucose metabolism may have a similar effect on β cell growth. Second, we found that on the HF diet, expression of Irs2 and Igf1r was upregulated in wild-type islets but markedly lower in the islets of Gck-/- mice (Table 1 and Figure 4, A–C), showing that Gck is required for the coordinated upregulation of Irs2 and Igf1r in the islets of HF diet–fed mice. Secondary effects of chronic hyper-
glycemia may also have contributed to the decreased Irs2 and Igf1r expression in the islets of Gck
 expression in the islets of Gck
 mice on the HF diet. It should be noted, however, that wild-type mice after 20 weeks on the HF diet and Gck
 mice on standard chow had similar glucose tolerance (Figure 1F) and that after another 20 weeks the HF diet-fed wild-type mice showed a greater increase in β cell mass, whereas even after 40 weeks on the HF diet the Gck
 mice showed only a small increase (Figure 2C). These results indicate that chronic hyperglycemia alone cannot fully explain the insufficient β cell hyperplasia of Gck
 mice on the HF diet. Third, in a previous study we showed a lack of β cell hyperplasia in response to genetically determined insulin resistance in Irs2
 mice (11), and in the present study we demonstrated that haploinsufficiency of Irs2 led to insufficient β cell hyperplasia on the HF diet (Figure 5, E and F). Fourth, we found that overexpression of Irs2 partially prevented diabetes in Gck
 mice on the HF diet. It should be noted, however, that the slight improvement in glucose tolerance can be explained by the fact that β cell mass, not β cell function, was restored in βIrs2TgGck
 mice compared with Gck
 mice on the HF diet (Figure 6, C–E). These results provide genetic evidence that Irs2 is critically involved in β cell hyperplasia on the HF diet. Based on these findings, we propose that both Gck and Irs2 are critical requirements for β cell hyperplasia in response to HF diet-induced insulin resistance.

What is the mechanism of Irs2 upregulation on the HF diet? We previously reported that the increase in intracellular calcium concentration in response to glucose was impaired in islets of Gck
 mice (ref. 3 and our unpublished observations). In the present study we noted the impaired CREB Ser133 phosphorylation in Gck
 mice on the HF diet. Taken together with the fact that cAMP and calcium pathways trigger CREB Ser133 phosphorylation, thereby upregulating Irs2
Our present results suggest that the impaired calcium signaling caused by haploinsufficiency of Gck in combination with HF diet–induced insulin resistance leads to the impaired Ser133 phosphorylation of CREB. Further study is needed to test this hypothesis.

What, then, is the molecular link between Irs2 and β cell proliferation? We noted the decreased FoxO1 nuclear exclusion in Gck+/– mice on the HF diet compared with wild-type mice on the HF diet (Figure 8, A and B). This finding, together with previous reports of a role of FoxO1 downstream of Irs2 in linking insulin signaling to Ipf1 regulation of β cells and compensation to insulin resistance (30, 31), suggests that decreased FoxO1 nuclear exclusion contributes to the insufficient proliferative response of existing β cells to insulin resistance in Gck+/– mice on the HF diet, and the fact that overexpression of Irs2 in β cells stimulated FoxO1 nuclear exclusion in wild-type and Gck+/– mice (Figure 8, A and B) is consistent with this hypothesis. Moreover, we noted lower Ipf1 nuclear expression in the β cells of Gck+/– mice on the HF diet than in those of wild-type mice on the HF diet (Supplemental Figure 1), which can be explained by the decreased FoxO1 nuclear exclusion in Gck+/– mice on the HF diet, as previously demonstrated in Irs2−/− mice (30). However, because haploinsufficiency of Ipf1 led to impaired β cell function, but not to decreased β cell mass (32), whether decreased Ipf1 nuclear expression is involved in the decreased β cell hyperplasia in Gck+/– mice on the HF diet remains unresolved.

To determine whether Ipf1 is involved in the regulation of β cell mass in Gck+/– mice, we are now investigating the phenotypes obtained by crossing Gck+/– mice with β cell–specific Ipf1 transgenic mice. Recently, β cell–specific ablation of Pdpk1 has been shown to induce diabetes as a result of loss of β cell mass (33); in the present study, expression of Pdpk1 actually decreased in HF diet–fed Gck+/– mice compared with wild-type mice (Table 1). Thus, inadequate acti-
viation of Pdkp1 downstream of Irs2 signaling may also play a role in the insufficient compensatory β cell hyperplasia in Gck\textsuperscript{+/–} mice on the HF diet. Interestingly, while expression of cyclin D1, cyclin D3, CDK4, and cyclin-dependent kinase inhibitors including p21 and p27 Kip1 was unaltered in the islets of Gck\textsuperscript{+/–} mice on the HF diet compared with those of wild-type mice on the HF diet, expression of cyclin D2 was downregulated (5.8-fold decrease) and expression of p15 inhibitor was slightly upregulated (1.2-fold increase). The roles of these cell cycle–related molecules in β cell proliferation downstream of Irs2 signaling should be examined in a future study.

What is the relationship between glucose metabolism and β cell proliferation? It has been established that β cell proliferation is not directly linked to glucose metabolism (4). Glucose oxidation can be explained by glucose metabolism. Because a HF diet is one of the environmental factors (34, 36). Because a HF diet is one of the environmental factors (34, 36).

Figure 8
Nuclear FoxO1-positive cells increased in Gck\textsuperscript{+/–} mice on the HF diet compared with wild-type mice on the HF diet, and overexpression of Irs2 in β cells decreased nuclear FoxO1-positive cells in Gck\textsuperscript{+/–} mice on the HF diet. (A) Immunohistochemical analysis of FoxO1 in islets from wild-type mice, βIrs2Tg mice, Gck\textsuperscript{+/–} mice, and βIrs2TgGck\textsuperscript{+/–} mice after 20 weeks on the HF diet. Representative islets are shown. FoxO1-positive cells are stained brown. Original magnification, ×600. (B) Ratio of the number of nuclear FoxO1-positive cells to the total number of islet cells. n = 43 (wild-type, Gck\textsuperscript{+/–}), 23 (βIrs2Tg), 48 (βIrs2TgGck\textsuperscript{+/–}). Values represent mean ± SEM. **P < 0.01.

Figure 7
Impaired Ser133 phosphorylation of CREB in Gck\textsuperscript{+/–} mice on HF diet. (A) Western blot assay of Ser133-phosphorylated CREB (p-CREB) and total CREB levels in islets from wild-type and Gck\textsuperscript{+/–} mice after 20 weeks on standard chow or HF diet. (B) Quantitation of Ser133 phosphorylation of CREB in wild-type and Gck\textsuperscript{+/–} mice after 20 weeks on standard chow or HF diet. Results are shown as proportions of the intensity of the Ser133-phosphorylated CREB band to that of the total CREB band (n = 5). Ser133 phosphorylation of CREB was significantly impaired in Gck\textsuperscript{+/–} mice compared with wild-type mice on the HF diet. Values represent mean ± SEM. *P < 0.05.
pivotal factors in the etiology of insulin resistance and obesity, our results should be relevant to the diversity in diabetes severity among MODY patients. Furthermore, since type 2 diabetes patients with decreased insulin secretion have been shown to have reduced β cell mass (37), Gck+/– mice on a HF diet should serve as a good animal model to better understand the relationship between decreased insulin secretion and decreased β cell mass in type 2 diabetes. Gck activation via small-molecule Gck activators in combination with increased cAMP production via glucagon-like peptide 1 derivatives may be a powerful strategy for the treatment of the decreased insulin secretion and decreased β cell mass in type 2 diabetes. In conclusion, the results of our study support the concept that Gck regulates β cell mass as well as β cell function. Irs2 was found to be involved in Gck-mediated β cell hyperplasia in HF diet-fed mice. Identification of the mechanism linking Gck and Irs2 should lead to novel therapeutic strategies that will increase β cell mass to compensate for HF diet–induced insulin resistance and may increase the amount of islets (β cells) for islet transplantation.

Methods

Animals. Gck+/– mice (129/Sv, ICR, and C57BL/6J hybrid background) were generated as described previously (3). Because of the heterogeneous genetic background of the mice, male offspring derived from Gck+/– intercrosses were analyzed in this study. The Gck−/− and wild-type mice were fed standard chow until 8 weeks of age and were then given free access to either standard chow or a HF diet. The animal care and procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo. Animals were maintained by means of standard animal care procedures based on the institutional guidelines. Irs1−/− and Irs2−/− mice (CBA and C57BL/6J hybrid background) were generated as described previously (8, 11). The βIrs2Tg mouse lineage was established by fusing the 0.74-kb rat insulin II promoter to a mouse Irs2 cDNA (4 kb), microinjecting the purified NotI fragment into the pronucleus of eggs of fertilized C57BL/6J mice (CLEA Japan Inc.), and then crossing F1 offspring with C57BL/6J mice (Supplemental Figure 2, A and B). Male offspring littersmates derived from crosses between male Gck+/– mice and female βIrs2Tg mice were analyzed in the Irs2 rescue experiments (Supplemental Figure 2, C and D).

Measurement of serum and islet parameters. Glucose, insulin, triglyceride, and FFA levels were determined with a GluTest Pro kit (SANWA KAGAKU KENKYUSHO CO. LTD.), an insulin kit (Biotrak; Amersham Biosciences), and L-type TG M and NEFA C-test kits (Wako), respectively.

Diet protocol. The composition of the standard chow (CLEA Rodent Diet CE-2; CLEA Japan Inc.) was 50.7% (wt/wt) carbohydrate, 4.6% fat, 25.2% protein, 4.4% dietary fiber, 6.5% crude ash, 3.6% mineral mixture, 1% vitamin mixture, and 4% moisture. The HF diet was 32% safflower oil, 33.1% casein, 17.6% sucrose, 5.6% cellulose, 9.8% mineral mixture, 1% vitamin mixture, and 0.5% DL-methionine.

In vivo glucose homeostasis. Glucose and insulin tolerance tests were performed as described previously (39, 40).

Histologic and immunohistochemical analysis and determination of adipocyte size. Adipose tissue was fixed with formaldehyde, and 10-μm sections were cut, mounted on glass slides, and stained with hematoxylin and eosin. White adipocyte areas were measured in 300 or more cells per mouse in each group, as described previously (39).

Immunohistochemical analysis of the endocrine pancreas and estimation of β cell and non–β cell mass and individual β cell size. Isolated pancreata were immersion-fixed in Bouin’s solution at 4°C overnight. Tissue was then routinely processed for paraffin embedding, and 2-μm sections were cut and mounted on glass slides. The sections were double immunostained with guinea pig anti-porcine insulin antibody (diluted 1:200) and a cocktail composed of rabbit anti-porcine glucagon (diluted 1:200), rabbit anti-human somatostatin (diluted 1:800), and rabbit anti-human pancreatic polypeptide (diluted 1:800) antibodies (all from Dako). Images of pancreatic tissue, islet β cell, and islet non–β cell were captured on a computer through a microscope connected to a charge-coupled device camera (all from Olympus). The area of the β cells and non–β cells relative to the total area of pancreatic tissue was calculated with WinROOF software (version 5.0, Mitani Corp.) as described previously (41). More than 20 pancreatic sections from each animal, including representative sections of the head, body, and tail of the pancreas, were analyzed, and approximately 100 islets per mouse were counted in each group. Adjacent nonoverlapping fields were analyzed to obtain a true representation of average islet/β cell distribution throughout the pancreas. Individual β cell size was estimated by dividing the β cell area by the number of β cell nuclei in the area covered, as described previously (21). Maximum islet diameter was also calculated with WinROOF software. Immunostaining with anti-Itp1 antibody (42) was performed as described previously (43). FoxO1 was immunohistochemically analyzed with anti-FoxO1 antibody (Cell Signaling Technology).

BrdU incorporation analysis and PCNA staining. BrdU incorporation was analyzed as described previously (44). In brief, BrdU (100 mg/kg in saline; Sigma-Aldrich) was intraperitoneally injected, and the pancreas was removed 6 hours later. The sections were double immunostained with anti-BrdU antibody (diluted 1:200; Dako) and a cocktail of anti-glucagon, anti-somatostatin, and anti-pancreatic polypeptide antibodies. BrdU-positive β cells were quantitatively assessed as a proportion of all β cells by counting the cells in approximately 50 islets per mouse. Sections were immunostained for PCNA with mouse anti-PCNA antibody (diluted 1:10; Progen Biotechnik) at 4°C for 48 hours.

Detection of apoptotic cells. Single-strand DNA analysis in islets was performed as described previously (45). Apoptotic cells were also detected in deparaffinized pancreatic sections by using an in situ cell death detection kit (Roche Diagnostics) according to the manufacturer’s recommendations.

Islet isolation. For metabolic analysis, islets were isolated with collagenase as described previously (46). For preparation for RNA or protein, islets were isolated by using liberase RI (Roche Diagnostics) according to the manufacturer’s instructions.

Analysis of insulin secretion and determination of glucose-phosphorylating activity, glucose oxidation, and cAMP content. Although the cellular composition of the islets may be affected by the dietary regimen, we used islets of comparable size (less than 250 μm in diameter) to assess islet insulin secretion and glucose metabolism, because quantitative determination of β cell mass in islets less than 250 μm in diameter yielded values in the 4 mouse groups that were indistinguishable. Insulin secretion by islets was analyzed as described previously (46). Glucose phosphorylation by hexokinase and Gck was assayed fluorometrically as described previously (3, 47). Hexokinase activity was measured at a glucose concentration of 0.5 mM, and the Gck activity was estimated as the difference between activity at 0.5 mM glucose and activity at 50 mM glucose. [U-14C]Glucose oxidation in mitochondria was assayed by measuring [14C]CO2 production as described previously (46). To extract cAMP for measurements, islet cells were disrupted by sonication for 5 seconds in 500 μl 95% ethanol at 4°C, vortexed vigorously, and centrifuged at 18,000 g for 30 minutes at 4°C. After removing the supernatant and evaporating to dryness, the samples were redissolved in sodium acetate buffer (0.05 mol/l, pH 6.2), and cAMP levels were determined with an enzyme-linked immunosorbent assay kit (cAMP Biotrak Enzymeimmunoassay System; Amersham Biosciences) in 96-well plates on a spectrophotometer at 450 nm according to the manufacturer’s instructions.

Microarray analysis of mRNA levels in isolated islets. Isolated islets were cultured overnight in RPMI 1640 medium containing 11.1 mM glucose (Sigma-Aldrich) supplemented with 10% FBS, 0.075 g/l penicillin (Sigma-Aldrich),...
and 0.1 g/1 streptomycin (Sigma-Aldrich). Total RNA was isolated with the RNeasy Mini Kit (QIAGEN) and used as starting material for cDNA preparation. RNA was prepared from 7 mice of identical genotype. The first- and second-strand cDNA synthesis, array hybridization, and scanning were performed as described previously (48). In brief, RNA amplification was started with 5 μg of total islet RNA. Double-stranded cDNA was synthesized with the SuperScript Choice system (Gibco) and a T7-(dT) 24 Primer (Amersham Pharmacia Biotech). In vitro transcription was performed to produce biotin-labeled cRNA by using a BioArray HighYield RNA Transcript Labeling Kit (Affymetrix) according to the manufacturer’s instructions. cRNA was linearly amplified approximately 40-fold with T7 polymerase by using half of the double-stranded cDNA that was synthesized. The readings obtained by quantitative scanning were analyzed with Affymetrix Gene Expression Analysis software. For expression change calls, we used the comparative analysis (Wilcoxon signed rank test–based analysis) program in Microarray Suite version 5.0 as described previously (49). The analysis was performed with the default parameters, where the P value of significant difference was below 0.0025.

**Immunoblotting.** The polyclonal anti-Irs1, anti-Irs2, and anti-p85 antibodies were purchased from Upstate USA Inc. The polyclonal anti-Inslr antibody, and anti–Igf1r1 antibody were purchased from Santa Cruz Biotechnology Inc. The anti-Akt antibody, anti-Igf1r1 antibody, anti-CREB antibody, and phospho-CREB (Ser133) antibody were purchased from Cell Signaling Technology Inc. The polyclonal anti-Ipf1 antibody (42) was provided by Y. Kajimoto (Osaka University, Suita, Japan). Islets were sonicated in ice-cold buffer A (25 mM Tris-HCl, pH 7.4, 10 mM Na3VO4, 10 mM NaPpi, 100 mM NaF, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) with an ultrasonic sonicator. Samples were prepared by SDS-polyacrylamide gel electrophoresis, and immunodetection was performed with an ECL kit (Amersham Biosciences). Protein was prepared from more than 100 islets pooled from several mice of identical genotype, and 20 μg samples of proteins were applied to the gel.

**Statistics.** Results are expressed as mean ± SEM. Statistical analysis was performed using the StatView software system (version 4.5; Abacus). Differences between 2 groups were analyzed for statistical significance by Student’s t test for unpaired comparisons. Individual comparisons among more than 2 groups were assessed with the post-hoc Fisher’s pairwise least-significant-difference test. A P value less than 0.05 was considered to be statistically significant.

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