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Functional adenosine triphosphate-sensitive potassium channel is required in high-carbohydrate diet-induced increase in β-cell mass

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ABSTRACT
Aims/Introduction: A high-carbohydrate diet is known to increase insulin secretion and induce obesity. However, whether or not a high-carbohydrate diet affects β-cell mass (BCM) has been little investigated.

Materials and Methods: Both wild-type (WT) mice and adenosine triphosphate-sensitive potassium channel-deficient (Kir6.2KO) mice were fed normal chow or high-starch (ST) diets for 22 weeks. BCM and the numbers of islets were analyzed by immunohistochemistry, and gene expression levels in islets were investigated by quantitative real-time reverse transcription polymerase chain reaction. MIN6-K8 β-cells were stimulated in solution containing various concentrations of glucose combined with nifedipine and glimepiride, and gene expression was analyzed.

Results: Both WT and Kir6.2KO mice fed ST showed hyperinsulinemia and body weight gain. BCM, the number of islets and the expression levels of cyclinD2 messenger ribonucleic acid were increased in WT mice fed ST compared with those in WT mice fed normal chow. In contrast, no significant difference in BCM, the number of islets or the expression levels of cyclinD2 messenger ribonucleic acid were observed between Kir6.2KO mice fed normal chow and those fed ST. Incubation of MIN6-K8 β-cells in high-glucose media or with glimepiride increased cyclinD2 expression, whereas nifedipine attenuated a high-glucose-induced increase in cyclinD2 expression.

Conclusions: These results show that a high-starch diet increases BCM in an adenosine triphosphate-sensitive potassium channel-dependent manner, which is mediated through upregulation of cyclinD2 expression.

INTRODUCTION
Insulin is secreted from pancreatic β-cells and plays an important role in maintaining glucose homeostasis. Pancreatic β-cell mass (BCM) is known to expand to compensate for increase in insulin demand. Indeed, BCM is increased in various rodent models, such as partial pancreatectomy and pregnancy.

Glucose is transported into the pancreatic β-cells through the glucose transporter and then metabolized to increase the adenosine triphosphate (ATP) concentration in β-cells. The increment of ATP concentration closes the ATP-sensitive potassium (KATP) channels, which consist of the Kir6.2 and sulfonylurea receptor 1 subunits. Closure of KATP channels induces depolarization of the β-cell membrane and allows Ca²⁺ flux through voltage-dependent calcium channels, which induces insulin secretion. It has been reported that...
intravenous glucose infusion for 2–7 days increases BCM in rats. Although proliferation of β-cells and/or acinar cell transdifferentiation into β-cells is considered to be involved in the increase of BCM, whether the KATP channel, which plays an essential role in glucose-induced insulin secretion (GIIS), participates in glucose-induced increase of BCM is not known well. We have previously reported that wild-type (WT) mice fed a high-starch diet (ST), the final product of which is glucose, show body weight gain and higher plasma insulin levels compared with mice fed normal chow (NC) 15 weeks after the intervention of diets despite a mild decrease in insulin sensitivity. In addition, GIIS is involved in the increase in BCM. For this purpose, we further analyzed the islets of fed ST. As BCM was increased in mice fed ST, we further investigated whether GIIS is involved in the increase of BCM. For this purpose, we further analyzed the islets of WT and KATP channel-deficient mice (Kir6.2KO), which showed complete defects in GIIS in vivo. The results show that feeding with ST increases BCM due to an increase in the number of islets in a KATP channel-dependent manner.

**METHODS**

**Animal and materials**

Kir6.2KO mice were generated as previously described. The Kir6.2KO mice had been backcrossed to the C57BL/6j mouse strain for more than five generations, and C57BL/6j (WT) mice were used as the control. Both mice were housed in a room under a standard 12-h light–dark cycle with free access to food and water. Eight-week-old male WT and Kir6.2KO mice were divided into two groups: mice fed NC (carbohydrate consisting of starch 58%, protein 29% and fat consisting of soy oil 13% of total energy) and ST (starch 74%, protein 13%, soy oil 13% of total energy; CLEA Japan, Osaka, Japan) for 22 weeks, and pancreata were excised for analyses at the end-point of the experiment. All animal experiments were carried out according to a protocol approved by the Nagoya University Institutional Animal Care and Use Committee.

**Plasma biochemical analyses**

Blood glucose levels were measured with Antsense Duo (Horiya, Kyoto, Japan), and plasma insulin levels were measured using a Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Kanagawa, Japan) as previously reported.

**In Vitro insulin secretion analysis**

MIN6-K8 β-cells were incubated for 30 min in Krebs–Ringer buffer containing 2.8 mmol/L glucose, and then stimulated for 240 min by 2.8 mmol/L glucose, 16.7 mmol/L glucose, 16.7 mmol/L glucose plus 10 μmol/L nifedipine (Wako, Osaka, Japan) or 2.8 mmol/L glucose plus 100 μmol/L glimepiride (Wako). In some experiments, cells were incubated in the presence of an insulin receptor antagonist, S961 (Phoenix Pharmaceuticals, Burlingame, CA, USA), at the concentration of 100 nmol/L throughout the experiments. Released insulin and insulin content were measured by an HTRF Insulin Kit (Cisbio Bioassays, Codolet, France), as previously reported.

**Insulin content analysis**

Insulin content of pancreas or MIN6-K8 β-cells was determined as previously described. Pancreatic tissue or MIN6-K8 cells were homogenized in Krebs–Ringer buffer (pH 7.4) on ice, and these homogenates were extracted overnight in acid-ethanol (1.5% [v/v] HCl in 75% [v/v] EtOH). Insulin contents in diluted extracts were measured by HTRF Insulin Kit (Cisbio Bioassays). Pancreatic insulin content was corrected by wet tissue weight for analysis.

**Isolation of ribonucleic acid and quantitative real-time reverse transcription polymerase chain reaction**

Mouse pancreatic islets were isolated using the collagenase digestion method. Total ribonucleic acid (RNA) was collected from isolated islets or MIN6K8 β-cells using the RNeasy Plus Kit (Qiagen, Tokyo, Japan); complementary deoxyribonucleic acid synthesis and quantitative real-time polymerase chain reaction were carried out as previously reported. Primer sequences are listed in Table S1. The messenger RNA (mRNA) levels were normalized by those of β-actin mRNA.

**Western blot analysis**

MIN6-K8 β-cells were stimulated for 20 h by 2.8 mmol/L glucose, 16.7 mmol/L glucose, 16.7 mmol/L glucose plus 10 μmol/L nifedipine or 2.8 mmol/L glucose plus 100 μmol/L glimepiride. Dispersed MIN6-K8 β-cells were sonicated in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitors [Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland]). Lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millpore, Billerica, MA, USA). The membranes were blocked in blocking solution (Tris-buffered saline [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl] containing 0.1% Tween 20 contained 1% bovine serum albumin) and incubated with primary antibodies against mouse anti-Actin (1:5,000; CP01; CALBIOCHEM, San Diego, CA, USA) or mouse anticyclin D2 (1:500; MA1-12297; Invitrogen, Carlsbad, CA, USA). Immunoreactivity was visualized with an enhanced chemiluminescence system, ECL Prime detecting reagents (GE Healthcare, Little Chalfont, UK) and detected by ImageQuant LAS 4000mini (GE Healthcare).
**Immunohistochemistry and morphometric analysis**

WT and Kir6.2KO mice were intraperitoneally injected with 5-bromo-2′-deoxyuridine (BrdU; Cosmo Bio Co., Ltd, Tokyo, Japan; 100 mg/kg body weight) 22 weeks after the intervention of the diets, and the whole pancreas was removed 6 h later. The pancreata of WT and Kir6.2KO mice were fixed in 4% paraformaldehyde and then embedded in paraffin. Serial sections of 4-μm thickness were cut from each paraffin block at 200-μm intervals and deparaffinized as previously reported. Sections were incubated overnight at 4°C with primary antibodies against insulin (1:300; ab7582; Abcam, Cambridge, MA, USA) or BrdU (1:200; ab6326; Abcam), followed by 90-min incubation in Alexa Fluor-conjugated secondary antibody (1:500; A11074; Alexa Fluor 546; Invitrogen, Grand Island, NY, USA) or (1:500; ab150157; Alexa Fluor 488; Abcam) at room temperature. The total areas of insulin-positive cells (β-cells) and the number of islets in six sections were analyzed using BZ-9000 fluorescent microscope system (Keyence, Osaka, Japan).

**Statistical analysis**

The results are presented as mean ± standard error of the mean. Statistical significance was evaluated by ANOVA or Student’s t-test using GraphPad Prism 7 for Windows (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

**WT mice fed ST showed body weight gain and higher plasma insulin levels**

We first investigated body weight, blood glucose levels and plasma insulin levels in WT mice fed NC or ST. A significant difference in body weight was apparent after 12 weeks of feeding and thereafter (Figure 1a). Blood glucose levels and plasma insulin levels under an ad libitum-fed condition were significantly higher in WT mice fed ST 15 weeks after the intervention of the diets compared with those in NC-fed WT mice (Figure 1b,c). Body weight was significantly heavier and blood glucose levels, under an ad libitum-fed condition, were significantly higher in WT mice fed ST compared with those in NC fed WT mice 22 weeks after the intervention of the diets (Figure S1a,b). Pancreatic insulin contents were significantly higher in ST-fed WT compared with those in NC-fed WT 22 weeks after the intervention of the diets (Figure 1d).

**Pancreatic islet number and BCM were increased in ST-Fed WT mice 22 weeks after intervention**

To address the effect of a high-carbohydrate diet on islets, we next investigated the morphology of islets in WT-fed NC or ST at the end-point of animal experiments, which was 22 weeks after the intervention of the diets. Both BCM and islet number were increased in ST-fed WT mice compared with that in NC-fed WT mice (Figure 2a–c). Mean islet size in ST-fed WT mice was smaller, although not significantly, compared with that in NC-fed WT mice (Figure 2d). We next investigated the relative number of islets and β-cells area in WT mice. The number of smaller size islets (<200 μm diameter) was increased in ST-fed WT mice compared with that in NC-fed mice; however, the number of larger size islets (>200 μm diameter) was not different between NC-fed and ST-fed WT mice (Figure 2e). To investigate β-cell proliferation, we carried out double staining for BrdU and insulin in the pancreatic sections (Figure 2f, g). The proportion of the double-positive cells was higher, although not significantly, in WT mice fed ST compared with that in WT mice fed NC.

**Expression levels of cyclin D2 mRNA in islets were higher in ST-fed WT mice 22 weeks after intervention**

We then analyzed the gene expression levels in islets of NC- and ST-fed WT mice. The expression levels of genes involved in insulin synthesis (insulin2), the insulin signal (Akt1) and the genes involved in the cell cycle (cyclinD1, D2 and cyclinE1) were significantly higher in the islets of ST-fed WT compared with those in the islets of NC-fed WT (Figure 3). In addition, the expression levels of insulin receptor substrate-2 (Irs2), which plays an important role in regulation of β-cell proliferation, showed a tendency to increase by ST feeding (Figure 3).

**Kir6.2KO mice fed ST also showed hyperinsulinemia and body weight gain**

To address whether functional KATP channels and/or GIIS are involved in ST-feeding-induced body weight gain, hyperinsulinemia and increase in BCM, we analyzed Kir6.2KO mice, in which GIIS is completely impaired. Kir6.2KO mice were fed NC or ST for 22 weeks. Body weights in Kir6.2KO mice fed ST were significantly heavier than those in Kir6.2KO mice fed NC after 13 weeks of feeding and thereafter, indicating that ST-induced body weight gain was in a KATP channel-independent manner (Figure 4a). Plasma insulin levels under an ad libitum-fed condition were significantly higher in Kir6.2KO mice fed ST compared with those in NC-fed Kir6.2KO mice 15 weeks after the intervention of the diets, although blood glucose levels were not different between the two groups (Figure 4b,c). Body weight was significantly heavier in Kir6.2KO mice fed ST compared with Kir6.2KO fed NC; however, blood glucose levels, under an ad libitum-fed condition, were not different between Kir6.2KO fed NC and ST 22 weeks after the intervention of the diets (Figure S1c,d). As these data are in parallel with those data in mice 15 weeks after the intervention of diets. Pancreatic insulin contents were not different between NC-fed Kir6.2KO mice and ST-fed Kir6.2KO mice 22 weeks after the intervention of the diets (Figure 4d).

**Pancreatic islet number and BCM were not increased in ST-fed Kir6.2KO mice 22 weeks after intervention**

We next investigated the morphology of islets in Kir6.2KO mice fed NC and ST. Neither BCM, the total number of islets, mean islet size nor the number of islets (<100 μm diameter; from 101 to 200 μm diameter; and >200 μm diameter) was
different between Kir6.2KO mice fed NC and ST (Figure 5a–e).

In addition, the proportion of the BrdU and insulin double-positive cells was not different between the two groups (Figure 5f,g).

**Expression levels of cyclin D2 mRNA in islets were not different between NC-Fed Kir6.2KO mice and ST-Fed Kir6.2KO mice 22 weeks after intervention**

We then analyzed the gene expression levels in islets of NC- and ST-fed Kir6.2KO mice. No significant difference in expression levels was observed in genes involved in insulin synthesis, the insulin signal or the cell cycle in Kir6.2KO mice fed ST compared with those fed NC (Figure 6).

**KATP channel-dependent pathway plays an important role in the mRNA expression levels of cyclin D2, but not of Irs2**

To address the role of the KATP channel in the regulation of gene expression in β-cells, we analyzed insulin secretion and gene expression in MIN6-K8 β-cells. Insulin secretion was significantly induced by 16.7 mmol/L glucose or 2.8 mmol/L glucose plus 100 nmol/L glimepiride (a KATP channel inhibitor, sulfonylurea), and GIIS was inhibited by nifedipine (an L-type calcium channel blocker) treatment (Figure 7a). The expression levels of *cyclinD2* and *Irs2* mRNA were increased by 16.7 mmol/L glucose (Figure 7b,c). The increase in *cyclinD2* mRNA expression was completely blocked by nifedipine treatment, whereas that in *Irs2* mRNA expression was marginally affected (Figure 7b,c). In contrast, glimepiride significantly increased the expression levels of *cyclinD2* mRNA, but did not affect the expression levels of *Irs2* mRNA at 2.8 mmol/L glucose concentration (Figure 7b,c), indicating that the KATP channel is involved in the regulation of the expression level of *cyclinD2*, but not *Irs2*. We next examined whether *cyclinD2* protein levels are regulated by a KATP channel-dependent pathway. Glucose significantly increased, and glimepiride tended to increase the expression levels of *cyclinD2* protein,
Figure 2 | Morphological analysis of islets in wild-type (WT) mice. (a) β-Cell area relative to pancreas area. (b) Number of islets relative to pancreas area. (c) The pancreatic section was stained with the antibody to insulin (red) and 4',6-diamidino-2-phenylindole (blue). Scale bars, 300 μm. (d) Distribution of islet sizes (*P < 0.05, **P < 0.01). (e) Mean islet size. (f) The percentage of 5-bromo-2'-deoxyuridine (BrdU)-positive β-cells. (g) The pancreatic section was double stained with the antibody to BrdU (green) and insulin (red) and 4',6-diamidino-2-phenylindole (blue). Scale bars, 100 μm (n = 6 per each group). Data are expressed as mean ± standard error of the mean. NC, normal chow; ST, high-starch diet.
and the cyclinD2 protein expression levels were decreased, but not significantly, by nifedipine treatment (Figure 7d,e). To address whether secreted insulin is involved in the regulation of the expression levels of cyclinD2 or Irs2, we pre-treated MIN6-K8 cells with S961, an insulin receptor antagonist, and then stimulated them with 16.7 mmol/L glucose concentration. Pretreatment with S961 did not block GIIS (Figure 7f). S961 also did not block the enhanced expression levels of cyclinD2 or Irs2 mRNA by 16.7 mmol/L glucose (Figure 7g,h).

**DISCUSSION**

In the present study, we showed that mice fed a high-carbohydrate (high starch) diet show increased BCM due to increased islet number. As these changes were not observed in Kir6.2KO mice, our data show that functional KATP channels are required in high-carbohydrate diet-induced increase in BCM.

It has been reported that BCM is reduced in mice deficient in cyclinD2 or Irs2, whereas BCM is increased in transgenic mice overexpressing Irs2 in a β-cell-specific manner. These reports show that cyclinD2 and Irs2 participate in the regulation of BCM. The expression levels of cyclinD2 are increased in liver-specific insulin receptor-deficient mice (LIRKO), which display hyperinsulinemia and insulin resistance. However, the increase in BCM is markedly attenuated in cyclinD2-deficient liver-specific insulin receptor-deficient mice. These results also underscore the important role of cyclinD2 in the increment of BCM under an insulin-resistant state.

Nutrients, such as glucose and fat, regulate β-cell proliferation. A high-fat diet (HFD) feeding induces body weight gain, hyperglycemia, hyperinsulinemia and increase in BCM within 7 days. An increase in the expression levels of cyclinD2 and Irs2 mRNA in islets has been documented in mice fed HFD for 20 weeks, suggesting that cyclinD2 and/or Irs2 contribute to HFD-induced increase in BCM. Furthermore, it has been reported that HFD-induced increase in BCM is attenuated in Irs2 heterozygous mice. In the present study, mice fed ST for 22 weeks showed body weight gain and hyperinsulinemia (Figure 1a,c). Additionally, BCM and the

![Figure 3](http://onlinelibrary.wiley.com/journal/jdi#fig3)
expression levels of cyclinD2 and Irs2 mRNA are increased in the islets of these mice (Figures 2 and 3). Whether or not the mechanism involved in ST-induced increase in BCM is distinct from that in HFD-induced increase should be further explored in future study. Nevertheless, we have found that the increased BCM in mice fed ST for 22 weeks is due to an increased number of small-to-medium size islets. These results concur with the report that 20% glucose infusion through the jugular vein for 48 h increases the number of smaller size islets in rats. Accordingly, an increase in the supply of glucose derived from ST might have contributed to the increase in the number of smaller size islets in the present study.

The mechanism involved in glucose-induced β-cell proliferation is not fully explored; however, a number of studies have shown that metabolism of glucose in β-cells, depolarization of the β-cell membrane and increase in intracellular Ca2+ concentration is involved.

Activation of glucokinase (GK), which catalyses the first step of glucose metabolism, increases the expression levels of cyclinD2 and Irs2 in rodent islets and insulinoma cell lines. In human cases with activating mutations of GK, persistent hyperinsulinemic hypoglycemia has been documented, and an increase in islet size has been observed at autopsy. Inhibition of KATP channel function by sulfonylurea has also been shown to induce β-cell proliferation. Sulfonylurea-induced β-cell proliferation is also observed in mice with β-cell-specific GK deficiency. In contrast, activation of the KATP channel by diazoxide suppresses β-cell proliferation induced by GK activation. These findings suggest that β-cell proliferation induced by GK activation is dependent on closure of the KATP channel, whereas β-cell proliferation induced by KATP channel closure is independent of GK activation. In the present study, incubation of MIN6-K8

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**Figure 4** | Changes in body weight, metabolic parameters and pancreatic insulin content in Kir6.2KO mice. (a) Changes in body weight in mice fed normal chow (NC; black circle; n = 8) or a high-starch diet (ST; black triangle; n = 8; *P < 0.05 compared with NC). (b) Blood glucose and plasma insulin levels under an ad libitum-fed condition. NC-fed mice (black bar; n = 8) and ST-fed mice (gray hatched bar; n = 8) 15 weeks after intervention (*P < 0.05). (c) Pancreatic insulin content in mice fed NC (black bar; n = 7) or ST (gray hatched bar; n = 7) 22 weeks after intervention. Data are expressed as mean ± standard error of the mean.
Figure 5 | Morphological analysis of islets in Kir6.2KO mice. (a) β-Cell area relative to pancreas area. (b) Number of islets relative to pancreas area. (c) The pancreatic section was stained with the antibody to insulin (red) and 4',6-diamidino-2-phenylindole (blue). Scale bars, 300 μm. (d) Distribution of islet sizes. (e) Mean islet size. (f) The percentage of 5-bromo-2'-deoxyuridine (BrdU)-positive β-cells. (g) The pancreatic section was double stained with the antibody to BrdU (green) and insulin (red) and 4',6-diamidino-2-phenylindole (blue). Scale bars, 100 μm (n = 6 per each group). Data are expressed as mean ± standard error of the mean.
β-cells in high-glucose media or with sulfonylurea increased cyclinD2 expression, whereas nifedipine attenuated a high-glucose-induced increase in cyclinD2 expression. In contrast, expression of Irs2 was increased by incubation in high-glucose media, but not by sulfonylurea, and a high-glucose-induced increase in Irs2 expression was not significantly inhibited by nifedipine in MIN6-K8 β-cells. These results are concordant with the report that sulfonylurea does not elevate the expression levels of Irs2 mRNA in islets of WT mice. Therefore, regulation of cyclinD2 expression is mediated through the KATP channel, but that of Irs2 expression is unlikely to be so mediated. These results suggest that closure of the potassium channel is insufficient to induce β-cell proliferation, and that the increase in intracellular Ca^{2+} concentration combined with an increase in the intracellular ATP:adenosine diphosphate ratio produced by glucose metabolism is required for β-cell proliferation.

It has been reported that insulin and downstream insulin signals participate in β-cell proliferation. However, it has been recently reported that insulin treatment and insulin receptor antagonist S961 do not affect β-cell proliferation in islets of mice. In addition, insulin treatment does not increase cyclinD2 expression levels in MIN6 cells. Consistent with these reports, S961 was found not to decrease the mRNA expression levels of cyclinD2 and Irs2 in MIN6-K8 β-cells under a high-glucose condition in the present study (Figure 7e,f).

Additionally, in the present study, GIIS was not induced during oral glucose tolerance test and intraperitoneal glucose tolerance test in Kir6.2KO mice fed ST (data not shown), as previously reported. Although Kir6.2KO mice are defective in GIIS, plasma insulin levels of Kir6.2KO are increased by ST feeding (Figure 4d). The present study has shown that functional KATP channels are required in ST-feeding-induced change in gene expression and increase in BCM, and suggests that a KATP
Figure 7 | Insulin secretion and change in messenger ribonucleic acid expression levels in MIN6-K8 β-cells. (a) Effects of glucose, glimepiride and nifedipine on insulin secretion in MIN6-K8 β-cells. Insulin secretion from MIN6-K8 β-cells was normalized by cellular insulin content (n = 7 per group). (b, c) Messenger ribonucleic acid expression levels of cyclinD2 and Irs2 in MIN6-K8 β-cells (n = 9 per group). (d) Representative immunoblots and (e) relative cyclin D2 protein expression levels normalized with respect to those of actin in MIN6-K8 β-cells (n = 12 per group). (f) Effect of S961 on insulin secretion in MIN6-K8 β-cells. Insulin secretion from MIN6-K8 β-cells was normalized by cellular insulin content (n = 12 per group). (g, h) Messenger ribonucleic acid expression levels of cyclinD2 and Irs2 in MIN6-K8 β-cells (n = 11–12; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data are expressed as means ± standard error of the mean. CCB, nifedipine; HG, 16.7 mmol/L glucose; LG, 2.8 mmol/L glucose; SU, glimepiride.
channel-independent signal, such as a vagal nerve signal, is insufficient to induce β-cell proliferation.

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DISCLOSURE

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Body weight and blood glucose levels in mice under an ad libitum-fed condition.
Table S1 | Primers used for quantitative real-time polymerase chain reaction.