Cephalic phase insulin secretion is \( K_{\text{ATP}} \) channel independent

Yusuke Seino\(^1\)\(^2\)\(^3\), Takashi Miki\(^1\)\(^4\), Wakako Fujimoto\(^1\), Eun Young Lee\(^4\), Yoshihisa Takahashi\(^1\), Kohtarō Minami\(^1\), Yutaka Oiso\(^3\) and Susumu Seino\(^1\)\(^5\)

\(^1\)Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
\(^2\)Departments of \(^3\)Metabolic Medicine and \(^3\)Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
\(^4\)Department of Medical Physiology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
\(^5\)Division of Diabetes, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Abstract

Glucose-induced insulin secretion from pancreatic \( \beta \)-cells critically depends on the activity of ATP-sensitive \( K^+ \) channels (\( K_{\text{ATP}} \) channel). We previously generated mice lacking \( \text{Kir}6.2 \), the pore subunit of the \( \beta \)-cell \( K_{\text{ATP}} \) channel (\( \text{Kir}6.2^{\text{C/C}} \)), that show almost no insulin secretion in response to glucose in vitro. In this study, we compared insulin secretion by voluntary feeding (self-motivated, oral nutrient ingestion) and by forced feeding (intra-gastric nutrient injection via gavage) in wild-type (\( \text{Kir}6.2^{\text{C/C}} \)) and \( \text{Kir}6.2^{\text{K/K}} \) mice. Under ad libitum feeding or during voluntary feeding of standard chow, blood glucose levels and plasma insulin levels were similar in \( \text{Kir}6.2^{\text{C/C}} \) and \( \text{Kir}6.2^{\text{K/K}} \) mice. By voluntary feeding of carbohydrate alone, insulin secretion was induced significantly in \( \text{Kir}6.2^{\text{K/K}} \) mice but was markedly attenuated compared with that in \( \text{Kir}6.2^{\text{C/C}} \) mice. On forced feeding of standard chow or carbohydrate alone, the insulin secretory response was markedly impaired or completely absent in \( \text{Kir}6.2^{\text{K/K}} \) mice. Pretreatment with a muscarine receptor antagonist, atropine methyl nitrate, which does not cross the blood–brain barrier, almost completely blocked insulin secretion induced by voluntary feeding of standard chow or carbohydrate in \( \text{Kir}6.2^{\text{K/K}} \) mice. Substantial glucose-induced insulin secretion was induced in the pancreas perfusion study of \( \text{Kir}6.2^{\text{K/K}} \) mice only in the presence of carbamylcholine. These results suggest that a \( K_{\text{ATP}} \) channel-independent mechanism mediated by the vagal nerve plays a critical role in insulin secretion in response to nutrients in vivo.

Key Words
- \( K_{\text{ATP}} \) channel
- cephalic phase insulin secretion
- acetylcholine

Introduction

Insulin secreted from pancreatic \( \beta \)-cells plays a pivotal role in the maintenance of glucose homeostasis. Insulin secretion is regulated by various factors including nutrients such as glucose, amino acids, and fatty acids; gastrointestinal hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinaotropic polypeptide (GIP); and neuronal (sympathetic and parasympathetic) inputs to \( \beta \)-cells (Nishi et al. 1987, Fehmann et al. 1995, Ahrén 2000, Gilon & Henquin 2001, Bratanova-Tochkova et al. 2002, Gautam et al. 2006, Nolan et al. 2006). Among these factors, glucose is unique in that it can trigger insulin secretion by itself, while the other factors stimulate or potentiate insulin only in the presence of glucose (Pagliara et al. 1975, Hedekov 1980, Weir et al. 1989, Henquin 2000). Glucose
is transported into the β-cells by glucose transporters and then metabolized, leading to an increase in the ATP concentration (ATP:ADP ratio), closure of the ATP-sensitive K⁺ (K\text{ATP}) channels, depolarization of the β-cell membrane, and opening of the voltage-dependent Ca²⁺ channels (VDCCs), which allows Ca²⁺ influx that triggers insulin secretion. The K\text{ATP} channel plays an essential role in this process by linking metabolic alteration to electrical activity of the cells.

We previously generated mice lacking Kir6.2, the pore subunit of the β-cell K\text{ATP} channel (Kir6.2\text{−/−}) (Miki et al. 1998), and found that Kir6.2\text{−/−} mice elicit no significant increase in insulin secretion in response to glucose using perfusion of isolated pancreatic islets (Miki et al. 1998) and perfusion of pancreas (Miki et al. 2005), both of which were performed under denervated, in vitro condition. In Sur1\text{−/−} mice, which lack the regulatory subunit of the channel, only a very small amount of insulin in response to glucose is triggered, as assessed by perfusion of pancreas (Shiota et al. 2002) and batch incubation of isolated pancreatic islets (Doliba et al. 2004). Recently, in pancreatic β-cell-specific Kir6.2 mutant mice, only a small amount of insulin in response to glucose was induced (Girard et al. 2009, Remedi et al. 2009). Intra-gastric glucose injection via gavage resulted in no or a very small insulin secretion in both Kir6.2\text{−/−} and Sur1\text{−/−} mice (Shiota et al. 2002, Miki et al. 2005). In addition to glucose, many other factors increase insulin secretion. Among these, GLP-1 and GIP potentiate insulin secretion in a glucose-dependent manner. We previously reported that there is an insulino-tropic effect of GLP-1 even under K\text{ATP} channel-deficient condition, suggesting a K\text{ATP} channel-independent mechanism of GIs; however, in Kir6.2\text{−/−} mice, GIs induced by GLP-1 is markedly attenuated when compared with that in the Kir6.2\text{+/-} mice (Fujimoto et al. 2009). In addition, acetylcholine stimulation was shown to increase intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ) in β-cells of both Kir6.2\text{−/−} and Sur1\text{−/−} mice in vitro (Miki et al. 1998, Doliba et al. 2004). In vivo, the insulin secretory response to mixed liquid meal via gastric gavage is delayed but is induced to some extent in Kir6.2\text{−/−} mice (Miki et al. 2005). However, Kir6.2\text{−/−} mice fail to develop diabetes in an ad libitum-fed state with standard chow. In addition, we incidentally found that plasma insulin levels did not differ in Kir6.2\text{+/-} and Kir6.2\text{−/−} mice in an ad libitum-fed state. These findings suggest that the insulin secretory response to glucose alone is defective in Kir6.2\text{−/−} mice, while that to other nutrition or vagal nerve stimulation may be maintained. In this study, we demonstrate that cephalic phase insulin secretion is mediated by neuronal action through the K\text{ATP} channel-independent pathway.

### Materials and methods

#### Mice

Kir6.2\text{−/−} mice were generated as described previously (Miki et al. 1998). As the Kir6.2\text{−/−} mice had been backcrossed to the C57BL/6 mouse strain for more than five generations, wild-type (Kir6.2\text{+/-}) C57BL/6 mice were used as control mice. The mice had free access to food and water for maintenance breeding. Prior to the voluntary feeding experiments, the mice were individually caged and trained for 3 days to immediately eat the test nutrients presented on the floor of the cage at mealtimes. All animal experiments were performed in accordance with the guidelines of the Animal Care Committee of Kobe and Chiba University.

#### Blood sampling

Blood glucose levels and plasma insulin levels were measured at 0, 15, and 60 min after nutrients were delivered.

#### Materials

Standard rodent normal chow diet, Racol (Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan), Twinline (Otsuka Pharmaceutical Co, Ltd), and dextrin were used. Standard normal chow (CE-2, CLEA Japan, Inc., Tokyo, Japan) contains 3.5 kcal/g calorie in total (protein 28.7%, carbohydrate 58.6%, and fat 12.7% kcal/g). A clinically used polymeric formula (Racol) mainly consists of casein from milk protein, soy protein isolate, maltodextrin, and purified white soft sugar and fat from oils and tricaprylin. It contains 5.06 g/dl protein, 16.2 g/dl carbohydrate, and 1.96 g/dl fat (1 kcal/ml calorie in total). The oligomeric formula (Twinline) comprises mostly di-and tri-peptides as nitrogen source, maltodextrin as carbohydrate, and safflower oil and tricaprylin as fat. It contains 4.05 g/dl protein, 14.7 g/dl carbohydrate, and 2.78 g/dl fat (1 kcal/ml calorie in total). Dextrin hydrate was purchased from Wako, Osaka, Japan. Racol, Twinline, or 30 g/dl dextrin were made into solid food by adding 4 g/dl agar.

#### Oral tolerance test

Into the stomach of mice, 1.5 ml dextrin liquid (containing dextrin hydrate 0.3 g) or 1.5 ml normal chow liquid (containing standard chow 0.3 g) were injected.
Voluntary feeding (re-feeding) and forced feeding experiment

Both Kir6.2−/− mice and Kir6.2+/+ mice were trained to eat the materials (standard chow, dextrin, Racol, or Twinline) as soon as they were put on the floor of the cage. After a 16-h fast, both Kir6.2−/− mice and Kir6.2+/+ mice were given either free access to the materials for voluntary feeding or they were injected directly into the stomach by gavage for the forced feeding experiment.

Atropine experiment

Sixty minutes before the mice ate standard chow or dextrin, either 2.5 mg/kg atropine methyl nitrate (Sigma; Havel et al. 1993) or saline was administered intraperitoneally.

Perfusion

Perfusion experiments using mouse pancreata were performed as described previously (Miki et al. 2005). Perfusion protocols began with a 10-min equilibration period with the same buffer used in the initial step. The insulin levels in the perfusate were measured by an ELISA kit (Mesacup Insulin Test) from BML, Inc. (Nagoya, Japan). Carbachol (carbamylcholine chloride) was from Wako.

Serum assay

Plasma insulin was measured using commercially available kits (Morinaga Institute of Biological Science, Yokohama, Japan).

Statistical analysis

Data are expressed as mean ± S.E.M. Significance of the differences between two groups was measured by Student’s t-test. ANOVA was used for multiple comparisons with a control group. A probability level of P<0.05 was considered statistically significant.

Results

Blood glucose and plasma insulin levels in voluntary feeding and forced feeding

Blood glucose and plasma insulin levels in fasted and ad libitum-fed states were similar in Kir6.2+/+ mice and Kir6.2−/− mice (Fig. 1a), indicating normal regulation of insulin secretion in physiological conditions in vivo. To evaluate the insulin response by voluntary feeding, we measured plasma insulin levels before and 15 and 60 min after the initiation of voluntary feeding of standard chow (CE-2). Plasma insulin levels in Kir6.2−/− mice were increased at 15 and 60 min to levels comparable to those of Kir6.2+/+ mice (Fig. 1b). These results show that the secretory response to voluntary feeding of standard chow in Kir6.2−/− mice is maintained.

We then administered powdered, water-suspended, standard chow directly into the stomach of Kir6.2+/+ mice or Kir6.2−/− mice by gavage. After forced feeding, insulin secretion in Kir6.2−/− mice at 15 min after loading was significantly lower than that in Kir6.2+/+ mice (62.6 ±10.2 pmol/l, n=4, 164 ±34.7 pmol/l, n=4, P<0.05) and the glucose level of Kir6.2−/− mice (33.0 ±2.85 mmol/l, n=4) was significantly higher than that of Kir6.2+/+ mice (21.0 ±2.38 mmol/l, n=4, P<0.05) at 60 min (Fig. 1c).

To assess the effects of feeding behavior (taste, mastication, and swallowing) on insulin secretion, we administered Kir6.2−/− mice dextrin, a partially hydrolyzed product of starch, by forced intra-gastric ingestion (0.3 g/body) or by voluntary feeding (23% (wt/vol) dextrin jellied by 4% agar). Dextrin is digested in the oral cavity by amylase and its derivative maltose gives rise to sweet sensation in the mouth (Ninomiya et al. 1998). Thus, voluntary feeding of dextrin is likely to evoke insulin secretion through sensing of sweetness in the cephalic phase of insulin secretion (Berthoud et al. 1980). Interestingly, a small but significant increase in insulin secretion was induced by voluntary feeding of carbohydrate only in Kir6.2−/− mice (Fig. 1d).

As expected, no significant increase in insulin secretion in Kir6.2−/− mice was detected by forced feeding of dextrin (carbohydrate alone) (Fig. 1e). In addition, we also found that voluntary feeding of dextrin augmented insulin secretion in Kir6.2−/− mice even at 60 min after dextrin ingestion, suggesting that cephalic regulation modulates both early (cephalic phase) and late (gut phase) phases of insulin secretion.

Participation of the parasympathetic nerve in insulin response to voluntary feeding

We then examined parasympathetic stimulation of GIs in KATP channel-deficient β-cells. We performed perfusion experiments in Kir6.2+/+ and Kir6.2−/− mice (Fig. 2a). In Kir6.2+/+ mice, 50 μM carbachol failed to stimulate insulin secretion in the presence of glucose (2.8 mM), but insulin secretion was markedly enhanced by switching
from 2.8 to 16.7 mM glucose (Fig. 2a, open circles). By contrast, in Kir6.2−/− mice, 50 μM carbachol significantly stimulated insulin secretion in the presence of 2.8 mM glucose, probably because the Ca2+ concentration in the presence of 2.8 mM glucose is already high in pancreatic β-cells of Kir6.2−/− mice (Miki et al. 1998).

Switching from 2.8 to 16.7 mM glucose stimulated insulin secretion significantly in pancreatic β-cells of Kir6.2−/− mice (Fig. 2a, solid circles). We then investigated involvement of the parasympathetic nerve in the insulin response to voluntary feeding in Kir6.2−/− mice using the muscarine receptor antagonist atropine, which does not cross the blood–brain barrier. We analyzed only mice eating more than 0.2 g standard chow within 15 min after initiation of voluntary feeding of standard chow. Pretreatment with atropine almost completely blocked the insulin response to voluntary feeding of standard chow in both Kir6.2+/+ and Kir6.2−/− mice (Fig. 2b). Pretreatment with...
atropine partially blocked the insulin response to voluntary feeding of dextrin in these Kir6.2<sup>C/C</sup> mice and completely blocked the insulin response to voluntary feeding of dextrin in these Kir6.2<sup>K/K</sup> mice (Fig. 2c). These findings demonstrate that parasympathetic neural input to β-cells is critical for the insulin response to voluntary feeding independent of the K<sub>ATP</sub> channel in vivo.

Effects of Twinline and Racol and insulin secretion

We then investigated the effects of nutrients other than carbohydrate on insulin secretion in vivo: the effects of voluntary feeding of Twinline jelled by 4% agar, which contains not only carbohydrate but also various lipids and amino acids, and Racol jelled by 4% agar, which contains carbohydrate, lipids, and polypeptides (Fig. 3a and b).
On the other hand, intake of Racol and Twinline in Kir6.2−/− mice induced insulin secretion to a level similar to that induced by dextrin alone (Fig. 3a and b) compared with Fig. 1d). These results indicate that only voluntary feeding of standard chow induces insulin secretion in Kir6.2−/− mice to a level similar to that of Kir6.2+/+ mice in vivo.

Nutrient and food intake

We found that intake of dextrin in voluntary feeding was greater in Kir6.2−/− mice than that in Kir6.2+/+ mice (Kir6.2−/−, 3.52 ± 0.223 g/120 min, n = 11, Kir6.2+/+, 2.69 ± 0.154 g/120 min, n = 10, *P < 0.01) (Fig. 4a). However, intake of standard chow by voluntary feeding in Kir6.2−/− mice was the same as that in Kir6.2+/+ mice. On the other hand, intake of Racol and Twinline in Kir6.2−/− mice was increased about 30% compared with that in Kir6.2+/+ mice, which is an increase similar to that by dextrin (Racol, 37.3 ± 6.38%, n = 5–6, *P < 0.01; Twinline, 24.3 ± 5.07%, n = 6–7, *P < 0.001).

We then evaluated the KATP channel-independent insulin response to voluntary feeding in the cephalic phase. We compared the increment of insulin secretion from 0 to 15 min in Kir6.2−/− mice with that in Kir6.2+/+ mice during voluntary feeding (Fig. 4b) and forced feeding (Fig. 4c). There was no difference in the insulin response to voluntary feeding of standard chow between Kir6.2+/+ mice and Kir6.2−/− mice, indicating that almost all of this insulin secretory response in Kir6.2−/− mice occurs in a KATP channel-independent manner. By contrast, the insulin response to voluntary feeding of Racol, Twinline, and dextrin was ~80% of that by the KATP channel-dependent pathway (Fig. 4b), and the insulin response to forced feeding of standard chow in Kir6.2−/− mice was decreased to ~20% in Kir6.2+/+ mice (Fig. 4c). Thus, the major pathway of cephalic phase insulin secretion by voluntary feeding of standard chow is KATP channel independent and regulated by parasympathetic nerves.

Discussion

The brain has long been known to respond to meal and elicit insulin secretion from β-cells through the CNS (Bergman & Miller 1973, Strubbe & Steffens 1975, Henderson et al. 1976, Louis-Sylvestre 1976, 1978, Steffens 1976, Powley 1977). The brain triggers a rapid but small increase in insulin secretion through autonomic input to β-cells. This response is followed by a long-lasting (up to 1 h), substantial secretory response mediated by nutrients in the gut lumen (Berthoud et al. 1980, 1981, Bellisle et al. 1985, Berthoud & Powley 1990). Cephalic phase insulin secretion can be evaluated only within an ~7-min period after meal ingestion because it is soon masked by the gut response to glucose.

We previously found that Kir6.2−/− mice almost completely lack GIIS (Miki et al. 1998). We show in this study that Kir6.2−/− mice retain normal insulin secretion to voluntary feeding of standard mouse chow but exhibit markedly attenuated insulin secretion by forced ingestion (Fig. 1b and c). In addition, the insulin secretory response to voluntary feeding of standard chow in Kir6.2−/− mice is completely blocked by atropine (Fig. 2b). We first considered the possibility that the atropine treatment inhibits gastrointestinal movement and digestion in the gut and therefore does not induce insulin secretion. However, blood glucose in atropine-treated
Kir6.2\(^{-/-}\) mice reached a level similar to that in saline-treated Kir6.2\(^{-/-}\) mice. Thus, insulin secretion during voluntary feeding of standard chow is mediated by parasympathetic input to \(\beta\)-cells. However, the insulin secretion induced by voluntary feeding of dextrin was partially blocked in Kir6.2\(^{+/+}\) mice in contrast to Kir6.2\(^{-/-}\) mice in which the insulin response was completely blocked. This indicates that the effect of dextrin-induced insulin secretion by voluntary feeding is mediated almost entirely through vagal nerve activity in Kir6.2\(^{-/-}\) mice whereas it is mediated only partially through vagal nerve activity in Kir6.2\(^{+/+}\) mice. This finding also indicates that various components of food induce insulin secretion through neural inputs to different degrees. Racol and Twinline contain fats and casein or amino acids in addition to carbohydrates. Voluntary feeding of Racol or Twinline in Kir6.2\(^{-/-}\) mice also did not elicit a sufficient amount of insulin secretion (Figs 3a, b, and 4b) (~20% of that in Kir6.2\(^{+/+}\) mice). Which factor in voluntary feeding of standard chow is the major determinant of insulin secretion is not known at present. Taste, smell, mastication, and visual information (Powley & Berthoud 1985, Suzuki et al. 2005) are implicated in normal insulin secretion in response to voluntary feeding of normal chow diet in Kir6.2\(^{-/-}\) mice.

A muscarine receptor subtype M3 is expressed in pancreatic \(\beta\)-cells and plays an important role in cholinergically mediated insulin secretion (Gautam et al. 2006). The cholinergic signal activates phospholipase C, which produces diacylglycerol and inositol 1,4,5-trisphosphate, thereby leading to insulin secretion through a rise in the intracellular \(Ca^{2+}\) concentration and protein kinase C activation in \(\beta\)-cells (Ahrén 2000, Gilon & Henquin 2001). This mechanism operates even in the K\(_{ATP}\) channel-deficient state in vivo. This accords with our previous in vitro finding that acetylcholine increases \([Ca^{2+}]\), to stimulate insulin secretion in Kir6.2\(^{-/-}\) islets (Miki et al. 1998).

In summary, there has been no good animal model in which cephalic phase insulin secretion can readily be distinguished from gut phase insulin secretion. Kir6.2\(^{-/-}\) mice exhibit no insulin secretion in response to gastric loading of standard chow, dextrin, or glucose but do exhibit insulin secretion in response to voluntary feeding of nutrients, which is completely blocked by atropine. In addition, insulin secretion in Kir6.2\(^{-/-}\) mice persists during voluntary feeding. These findings suggest that Kir6.2\(^{-/-}\) mice can be used to unmask neural involvement during gut phase insulin secretion.

**Figure 4**
(a) Differences in food intake of various nutrients between Kir6.2\(^{+/+}\) mice (white bar) and Kir6.2\(^{-/-}\) mice (black bar) (n = 5–11). The amount of food intake was calculated for 120-min refeeding after 16-h fasting. Asterisk (*) indicates a significant difference between Kir6.2\(^{+/+}\) mice and Kir6.2\(^{-/-}\) mice (**\(P<0.01\), ***\(P<0.001\)). (b) The increment of insulin secretion from 0 to 15 min in Kir6.2\(^{+/+}\) mice and Kir6.2\(^{-/-}\) mice during voluntary feeding. Kir6.2\(^{+/+}\) mice (white bar) and Kir6.2\(^{-/-}\) mice (black bar) (**\(P<0.01\), ***\(P<0.001\)). (c) The increment of insulin secretion from 0 to 15 min in Kir6.2\(^{+/+}\) mice and in Kir6.2\(^{-/-}\) mice during forced feeding. Kir6.2\(^{+/+}\) mice (white bar) and Kir6.2\(^{-/-}\) mice (black bar) (**\(P<0.01\), ***\(P<0.001\)).


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