Rapamycin impairs metabolism-secretion coupling in rat pancreatic islets by suppressing carbohydrate metabolism

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Abstract

Rapamycin, an immunosuppressant used in human transplantation, impairs β-cell function, but the mechanism is unclear. Chronic (24 h) exposure to rapamycin concentration dependently suppressed 16·7 mM glucose-induced insulin release from islets (1·65±0·06, 30 nM rapamycin versus 2·35±0·11 ng/islet per 30 min, control, $n=30$, $P<0·01$) without affecting insulin and DNA contents. Rapamycin also decreased α-ketoisocaproate-induced insulin release, suggesting reduced mitochondrial carbohydrate metabolism. ATP content in the presence of 16·7 mM glucose was significantly reduced in rapamycin-treated islets (13·42 ±0·47, rapamycin versus 16·04±0·46 pmol/islet, control, $n=30$, $P<0·01$). Glucose oxidation, which indicates the velocity of metabolism in the Krebs cycle, was decreased by rapamycin in the presence of 16·7 mM glucose (30·1±2·7, rapamycin versus 42·2±3·3 pmol/islet per 90 min, control, $n=9$, $P<0·01$). Immunoblotting revealed that the expression of complex I, III, IV, and V was not affected by rapamycin. Mitochondrial ATP production indicated that the respiratory chain downstream of complex II was not affected, but that carbohydrate metabolism in the Krebs cycle was reduced by rapamycin. Analysis of enzymes in the Krebs cycle revealed that activity of α-ketoglutarate dehydrogenase (KGDH), which catalyzes one of the slowest reactions in the Krebs cycle, was reduced by rapamycin (10·08±0·82, rapamycin versus 13·82 ±0·84 nmol/mg mitochondrial protein per min, control, $n=5$, $P<0·01$). Considered together, these findings indicate that rapamycin suppresses high glucose-induced insulin secretion from pancreatic islets by reducing mitochondrial ATP production through suppression of carbohydrate metabolism in the Krebs cycle, together with reduced KGDH activity.
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Introduction

Rapamycin, an immunosuppressant used in human organ and tissue transplantation, exhibits a different mechanism of action from that of cyclosporine, tacrolimus, and corticosteroids. The agent is a macrolide that prevents T-cell activation through its inhibitory effect on serine/threonine kinase, the mechanistic target of rapamycin (MTOR). The insulin- and nutrient-signaling pathway through MTOR plays an important role in initiation of protein translation, a critical event in enhanced protein synthesis that leads to increased cell cycle progression and proliferation (McDaniel *et al.* 2002).

Brittle type 1 diabetes has been successfully treated by human islet transplantation using the Edmonton protocol, which includes use of rapamycin as an immunosuppressant (Shapiro *et al.* 2000). Some studies suggest rapamycin may be diabetogenic (Lu *et al.* 1994, Teutonico *et al.* 2005), and the effects of rapamycin on glucose homeostasis have been investigated. In skeletal muscle cells, long-term exposure to rapamycin decreases insulin-dependent uptake of glucose and glycogen synthesis and increases fatty acid oxidation (Sipula *et al.* 2006). Rapamycin also decreases insulin-mediated glucose uptake and insulin signaling in adipocytes (Taha *et al.* 1999, Cho *et al.* 2004). Interestingly, rapamycin both prevents β-cell mass expansion and impairs β-cell function (Bell *et al.* 2003, Zhang *et al.* 2006, Fraenkel *et al.* 2008).

In pancreatic β-cells, intracellular glucose metabolism regulates exocytosis of insulin granules according to metabolism-secretion coupling in which glucose-induced mitochondrial ATP production plays an essential role (Maechler & Wollheim 2001). Since depletion of mitochondrial DNA abolishes the glucose-induced ATP elevation, mitochondria clearly are a major source of ATP production in pancreatic β-cells (Kennedy *et al.* 1998, Tsuruzoe *et al.* 1998). Glucose-induced insulin secretion from β-cells is often impaired due to reduced glucose-induced ATP elevation by exposure to high concentrations of fuels including glucose, free fatty acids, and ketone body, and by administration of diabetogenic pharmacological agents (Fujimoto *et al.* 2007). Thus, reduced mitochondrial ATP production plays an important role in impaired glucose-induced insulin secretion.

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Recently, several reports have shown that inhibition of MTOR by rapamycin decreases mitochondrial oxidative function using various materials including kidney mitochondria (Simon *et al.* 2003), Jurkat cells (Schieke *et al.* 2006), and skeletal tissue and cells (Cunningham *et al.* 2007). We investigated the effects of chronic exposure to rapamycin on metabolism-secretion coupling, especially on glucose metabolism in mitochondria, in pancreatic β-cells.

Materials and Methods

Materials

Rapamycin was purchased from Calbiochem (La Jolla, CA, USA). Disodium succinate, rotenone, pyruvate potassium, malate, and tetramethyl-*p*-phenyldiamine (TMPD) were purchased from Nacalai (Kyoto, Japan). Mouse monoclonal antibody to the subunits of the mitochondrial respiratory chain complex was obtained from Invitrogen. [5-^{3}H]-glucose, [U-^{14}C]-glucose, and anti-mouse IgG HRP-conjugated secondary antibody were obtained from GE Healthcare (Buckinghamshire, UK). Acetyl-CoA was obtained from Wako (Osaka, Japan). Luciferin–luciferase was obtained from Promega. All other reagents were obtained from Sigma Chemicals.

Animals

Male Wistar rats were obtained from Shimizu Co. (Kyoto, Japan). The animals were fed standard laboratory chow *ad libitum* and allowed free access to water in an air-conditioned room with a 12 h light:12 h darkness cycle until the experiments. All experiments were carried out with rats aged 8–11 weeks. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University.

Islet isolation and culture

Islets of Langerhans were isolated from Wistar rats by collagenase digestion as previously described (Fujimoto *et al.* 1998). Isolated islets were cultured for 24 h in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5·5 mM glucose with or without rapamycin, at 37 °C in humidified air containing 5% CO_2.

Measurement of insulin release from isolated rat pancreatic islets, insulin content, and DNA content

Insulin release from intact islets was monitored using batch incubation as previously described (Fujimoto *et al.* 1998) using Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 0·2% BSA (fraction V) and 10 mM HEPES adjusted to pH 7·4 (KRBB medium). After cultured islets were preincubated at 37 °C for 30 min in KRBB medium supplemented with 2·8 mM glucose, groups of five islets were batch incubated for 30 min in 0·7 ml KRBB medium containing 2·8 and 16·7 mM glucose with or without 100 μM α-tocopherol plus 200 μM ascorbate, or containing 2·8 and 16·7 mM α-ketoisocaproate (KIC). Before addition to KRBB medium, α-tocopherol was dissolved in ethanol at 1000-fold concentration. The same amount of ethanol was added to the control solution. At the end of the incubation period, the islets were pelleted by centrifugation, and aliquots of the buffer were sampled to determine the amount of immunoreactive insulin by RIA. After an aliquot of incubation medium for insulin release assay was taken, the islets remaining were lysed to determine insulin and DNA contents as previously described (Fujimoto *et al.* 2000).

Measurement of ATP content

ATP contents were determined as previously described (Kominato *et al.* 2008). Briefly, after groups of cultured islets were preincubated at 2·8 mM glucose for 30 min, groups of ten islets were incubated in tubes containing 0·5 ml KRBB medium supplemented with 2·8 or 16·7 mM glucose with or without 100 μM α-tocopherol plus 200 μM ascorbate at 37 °C for 30 min. Incubation was stopped by the addition of 0·1 ml of 2 M $HClO_4$. The contents of tubes were immediately mixed with vortex and sonicated in ice-cold water. The tubes were then centrifuged, and a fraction (0·4 ml) of the supernatant was mixed with 0·1 ml of 2 M HEPES and 0·1 ml of 1 M Na_2CO_3. The ATP concentration was measured by adding 0·2 ml luciferin–luciferase solution to a fraction sample (0·1 ml) in a bioluminometer (Luminometer Model 20e, Turner Designs, Sunnyvale, CA, USA). To draw a standard curve, blanks and ATP standards were run through the entire procedure including the extraction steps.

Measurement of glucose utilization and oxidation

Glucose utilization and oxidation were measured using the previously described method (Nabe *et al.* 2006). Briefly, cultured islets were preincubated in KRBB medium with 2·8 mM glucose at 37 °C for 30 min. For glucose utilization measurements, tubes containing 25 islets in 150 μl KRBB medium containing 2·8 or 16·7 mM glucose and 1·5 μCi [5-^{3}H] glucose were placed into glass vials containing 0·5 ml water. The capped vials were incubated at 37 °C for 90 min. After incubation was stopped by adding 50 μl of 1 M HCl into the incubation medium of the tubes without opening the caps, the capped vials were incubated overnight at 34 °C to allow $^{3}H_2O$ in the tubes to equilibrate with the water in the vial. Each tube was removed, and the disintegrations per minute of $^{3}H_2O$ in the water were counted. For oxidation measurements, procedures were the same as those for utilization measurements, except for the use of [U-^{14}C] glucose (0·5 μCi/tube) in place of [5-^{3}H] glucose and the use of 0·5 ml hydroxide of hyamine 10-X (Packard, Meriden, CT, USA) in place of 0·5 ml water.

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Measurement of glucokinase activity

Glucokinase activity was measured by a fluorometric assay as previously described (Radu *et al.* 2005). Briefly, after cultured islets were preincubated with KRBB medium with 2·8 mM glucose, 100 islets were homogenized and the supernatants (islet extracts) were obtained from the homogenates by centrifugation. The glucose phosphorylation rate was estimated as the increase in NADH through the following reaction: glucose-6-phosphate + NAD^+ → 6-phosphoglucono-δ-lactone + NADH by NAD^+-dependent glucose-6-phosphate dehydrogenase (G6PDH). The enzyme reaction was performed using islet extracts in a solution containing NAD^+ and G6PDH supplemented with two concentrations (50 and 0·5 mM) of glucose at 37 °C for 1 h. NADH concentration was measured by fluorometry (Shimazu RF-5000, Kyoto, Japan).Glucokinase activity was determined by subtracting hexokinase activity measured at 0·5 mM glucose from the activity measured at 50 mM glucose.

Measurement of mitochondrial ATP production

Measurement of ATP production from mitochondrial fraction was performed as previously described (Takehiro *et al.* 2005). Briefly, to measure ATP production by oxidative phosphorylation, the reaction was started by adding mitochondrial suspension to prewarmed solution (37 °C) supplemented with the mitochondrial substrates, 50 μM ADP, and 1 μM diadenosine pentaphosphate (DAPP). DAPP is a specific inhibitor of adenylate kinase used to measure ATP production by oxidative phosphorylation exclusively. To normalize the mass of the intact mitochondria obtained, ATP production by adenylate kinase, one of the mitochondrial intermembrane kinases, was measured in the presence of ADP but without mitochondrial substrates or DAPP in parallel incubations. After reaction was stopped, the ATP concentration in the solutions was measured by adding luciferin–luciferase solution with a bioluminometer. ATP production was determined as the ratio of ATP production by oxidative phosphorylation to that by adenylate kinase.

Western blotting of mitochondrial respiratory chain complexes

After washing with ice-cold PBS, the cultured islets were solubilized in ice-cold lysis buffer (10 mM Tris (pH 7·2), 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0·5% sodium deoxycholate) containing protease inhibitor cocktail (Complete; Roche) with sonication (5 s pulse, five times). Protein content of the supernatant was measured and adjusted by Bradford method. The supernatant was dissolved in the same amount of SDS-PAGE sample buffer containing 100 mM Tris–HCl (pH 6·80), 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and 1% bromophenol blue and boiled for 5 min at 95 °C. The samples were subjected to electrophoresis on 12% SDS–polyacrylamide gels and transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). After blocking with TBS containing 0·1% Tween 20 and 5% skimmed milk (blocking buffer) for 1 h at 4 °C, blotted membranes were incubated overnight at 4 °C with mouse monoclonal anti-complex I (39 kDa subunit), anti-complex III (core II), anti-complex IV (subunit I), or anti-complex V (subunit α) of mitochondrial respiratory chain antibody at 1:1000 dilution in blocking buffer, and subsequently with anti-mouse IgG HRP-conjugated secondary antibody diluted 1:5000 at room temperature for 1 h prior to detection using ECL (GE Healthcare). In the same membrane, the process was repeated for β-actin at 1:5000 dilution of the antibody. Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).

Measurement of activities of enzymes in Krebs cycle

Mitochondrial fraction obtained as described above was sonicated in ice-cold solution containing (mM) 180 KCl, 5 morpholinepropanesulfonic acid, and 2 EDTA adjusted to pH 7·40 and then diluted to each reaction mixture. Enzyme activities including NAD^+-linked isocitrate dehydrogenase (NAD-ICDH), aconitase, α-ketoglutarate dehydrogenase (KGDH), and malate dehydrogenase (MDH) were measured as previously described (Nulton-Persson & Szweda 2001). NAD-ICDH activities were measured as the rate of NAD^+ reduction in solution A containing (mM) 25 KH_2PO_4, 0·5 EDTA, and 0·01% Triton X-100 adjusted to pH 7·25 supplemented with 2·5 mM isocitrate, 40 μM rotenone, 5 mM $MgCl_2$, and 1 mM NAD^+. Aconitase activities were measured as the rate of $NADP^+$ reduction in solution A with 5 mM citrate, 0·6 mM $MgCl_2$, 1·0 U/ml NADP-ICDH, and 0·2 mM $NADP^+$. KGDH activities were measured as the rate of NAD^+ reduction in solution A with 2·5 mM α-ketoglutarate, 40 μM rotenone, 5·0 mM $MgCl_2$, 1 mM NAD^+, 0·1 mM CoA, and 0·2 mM thymine pyrophosphate (TPP). MDH activities were measured as the rate of NAD^+ reduction in solution A with 2·5 mM malate, 40 μM rotenone, 5 mM $MgCl_2$, 10 mM NAD^+, 0·3 mM acetyl-CoA, and 1 U/ml citrate synthase. Enzyme activities of pyruvate dehydrogenase (PDH) were measured as total PDH complex activity (Schwab *et al.* 2005) as the rate of *p*-iodonitrotetrazolium violet (INT) reduction in a reaction mixture containing 5 mM L-carnitine, 1·0 mM $MgCl_2$, 2·5 mM NAD^+, 0·1 mM CoA, 5 mM pyruvate, 0·2 mM TPP, 0·1% Triton X-100, 1 g/l BSA, 0·6 mM INT, and 6·5 mM phenazine methosulfate. All enzyme assays were performed at 25 °C.

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical significance was calculated by unpaired Student's *t*-test. $P < 0{\cdot}05$ was considered significant.

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Results

Effect of chronic exposure to rapamycin on glucose-induced insulin release, insulin content, and DNA content in islets

Chronic (24 h) exposure to rapamycin (10, 30, and 100 nM) concentration dependently suppressed 16·7 mM glucose-induced insulin release (1·94±0·09, 10 nM; 1·65±0·06, 30 nM; 1·50±0·06, 100 nM rapamycin versus 2·35 ±0·11 ng/islet per 30 min, control, $n=30$, $P<0·01$ respectively) but did not affect basal insulin release in the presence of 2·8 mM glucose (Fig. 1A). Insulin secretion divided by insulin content also demonstrates that rapamycin suppresses glucose-induced insulin secretion (Fig. 1A). Insulin and DNA contents were not affected by 24-h exposure to 10, 30, and 100 nM rapamycin (Table 1), indicating that these concentrations of rapamycin do not reduce islet β-cell mass. Reactive oxygen species (ROS) scavengers did not affect suppressed glucose-induced insulin secretion by rapamycin (1·60±0·10, 30 nM rapamycin versus 1·69±0·10 ng/islet per 30 min, 30 nM rapamycin with α-tocopherol plus ascorbate, $n=10$, not significant).

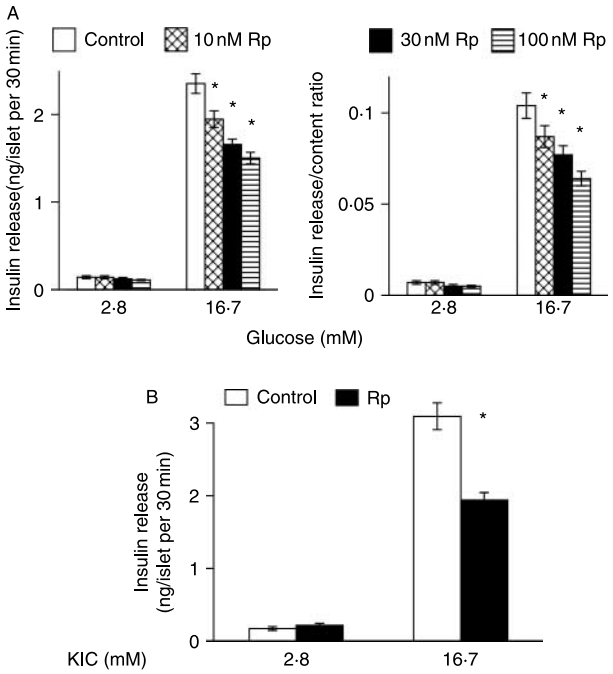

Figure 1 Effects of chronic exposure to rapamycin (Rp) on fuel secretagogue-induced insulin release from islets. (A) High (16·7 mM) glucose-induced and basal insulin release in control and Rp-treated islets. Islets were cultured with 10, 30, and 100 nM Rp or without Rp for 24 h. After cultured islets were preincubated with 2·8 mM glucose for 30 min, they were incubated with 2·8 and 16·7 mM glucose. Insulin secretions are presented as insulin secretion for 30 min/islet (right) and as the ratio of insulin secretion for 30 min to insulin content (left). Values represent mean±S.E.M. of 30 determinations. *$P<0·01$ versus corresponding control. (B) High KIC (16·7 mM)-induced and basal insulin release in control and Rp-treated islets. Islets were cultured with or without 30 nM Rp for 24 h. After cultured islets were preincubated with 2·8 mM glucose for 30 min, they were incubated with 2·8 and 16·7 mM KIC. Values represent mean±S.E.M. of 18 determinations. *$P<0·01$ versus corresponding control.

Table 1 Effect of chronic exposure to rapamycin on insulin content and DNA content. At the end of experiments indicated in Fig. 1A, insulin content and DNA content in islets were determined. Values represent mean±S.E.M. of 60 determinations

	Insulin content (ng/islet)	**DNA content** (ng/islet)
Experimental condition during culture		
Control	24·7±1·0	14·6±0·4
10 nM rapamycin	24·1±1·0	14·5±0·4
30 nM rapamycin	24·5±1·1	15·0±0·5
100 nM rapamycin	23·7±0·8	14·1±0·3

Effect of chronic exposure to rapamycin on KIC-induced insulin release

To characterize metabolic fuel-induced insulin release independent of glycolysis, KIC-induced insulin release from rapamycin-treated islets was examined. Chronic exposure to 30 nM rapamycin decreased high KIC-induced insulin release (1·93±0·10, rapamycin versus 3·09±0·18 ng/islet per 30 min, control, $n=18$, $P<0·01$; Fig. 1B).

Effect of rapamycin on ATP content

ATP content was greater in control islets incubated with 16·7 mM glucose than in control islets incubated with 2·8 mM glucose (11·97±0·35, 2·8 mM glucose versus 16·04±0·46 pmol/islet, 16·7 mM glucose, $n=30$, $P<0·01$; Fig. 2). ATP content in the presence of 16·7 mM glucose

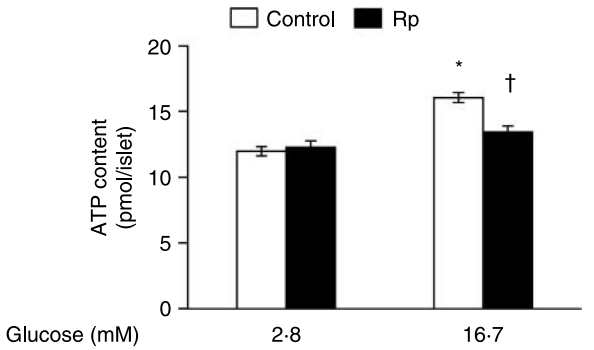

Figure 2 Effects of chronic exposure to rapamycin (Rp) on ATP contents in islets. Islets were cultured with or without 30 nM Rp for 24 h. After cultured islets were preincubated with 2·8 mM glucose for 30 min, and then incubated with 2·8 and 16·7 mM glucose for 30 min, ATP contents were determined. Values represent mean ±S.E.M. of 30 determinations. *$P<0·01$ versus control with 2·8 mM glucose. †$P<0·01$ versus control with 16·7 mM glucose.

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was significantly reduced in rapamycin-treated islets (13·42 ±0·47 pmol/islet, 16·7 mM glucose, rapamycin versus 16·7 mM glucose, control, $n=30$, $P<0·01$), but that in the presence of 2·8 mM glucose was not affected by rapamycin (Fig. 2). ROS scavengers did not affect the suppressed ATP content in the presence of high glucose by rapamycin (13·27 ±0·92, 30 nM rapamycin versus 14·58±0·82 pmol/islet, 30 nM rapamycin with α-tocopherol plus ascorbate, $n=10$, not significant).

Effects of rapamycin on glucose utilization and glucose oxidation

Glucose utilization was greater in islets incubated with 16·7 mM glucose than that in islets incubated with 2·8 mM glucose in both control (33·0±1·8, 2·8 mM glucose versus 98·4±5·0 pmol/islet per 90 min, 16·7 mM glucose, $n=15$, $P<0·01$) and rapamycin-treated islets (28·1±1·7, 2·8 mM glucose versus 75·1±2·6 pmol/islet per 90 min, 16·7 mM glucose, $n=15$, $P<0·01$). Glucose utilization in the presence of 16·7 mM glucose was significantly reduced in rapamycin-treated islets ($P<0·01$), but that in the presence of 2·8 mM glucose was not affected by rapamycin (Fig. 3A).

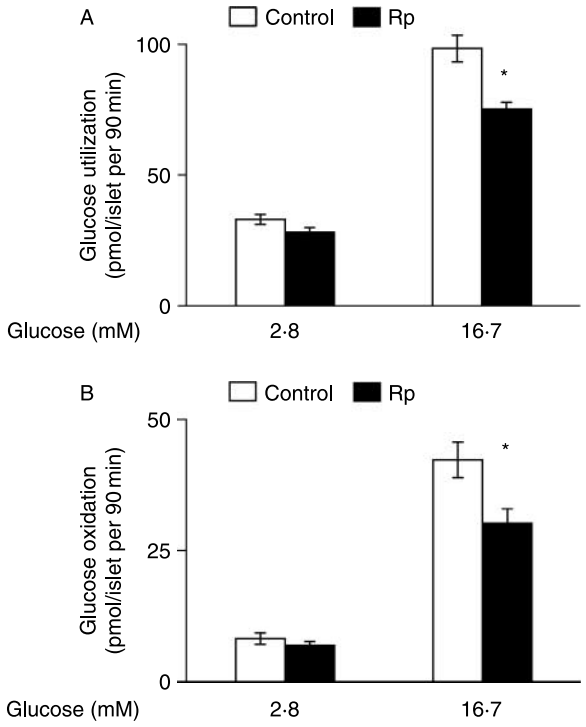

Figure 3 Effects of chronic exposure to rapamycin (Rp) on glucose utilization and oxidation in islets. Islets were cultured with or without 30 nM Rp for 24 h. After cultured islets were preincubated with 2·8 mM glucose for 30 min, they were incubated with 2·8 and 16·7 mM glucose for 90 min. (A) Glucose utilization. Values represent mean±S.E.M. of 15 determinations. *$P<0·01$ versus corresponding control. (B) Glucose oxidation. Values represent mean±S.E.M. of nine determinations. *$P<0·01$ versus corresponding control.

Glucose oxidation was greater in islets incubated with 16·7 mM glucose than that in islets incubated with 2·8 mM glucose in both control (8·1±1·1, 2·8 mM glucose versus 42·2±3·3 pmol/islet per 90 min, 16·7 mM glucose, $n=9$, $P<0·01$) and rapamycin-treated islets (6·8±0·7, 2·8 mM glucose versus 30·1±2·7 pmol/islet per 90 min, 16·7 mM glucose, $n=9$, $P<0·01$). Glucose oxidation in the presence of 16·7 mM glucose was significantly reduced in rapamycin-treated islets ($P<0·01$), but that in the presence of 2·8 mM glucose was not affected by rapamycin (Fig. 3B). Glucose oxidation in the presence of 16·7 mM glucose declined 77% by 100 nM antimycin A, which is comparable with the reduction by rapamycin treatment. In the same condition, glucose utilization with high glucose also declined 78% by antimycin A (Table 2).

Effect of rapamycin on glucokinase activity

Glucokinase activity was not affected by rapamycin treatment (87·4±10·4, rapamycin versus 75·4±14·8 pmol/islet per 60 min, control, $n=3$, not significant).

Effect of rapamycin on expression of mitochondrial respiratory chain complexes

Immunoblotting using lysates of whole islets revealed that rapamycin did not affect expression of complex I, III, IV, and V of the mitochondrial respiratory chain proteins (Fig. 4).

Effect of rapamycin on ATP production by mitochondria from islets

ATP production by mitochondria from control and rapamycin-cultured islets in the presence of various substrates and inhibitors is shown in Table 3. Antimycin A, a complex III inhibitor in the respiratory chain, inhibited ATP production dramatically in the presence of succinate in mitochondria from both control and rapamycin-cultured islets. Mitochondrial ATP production of rapamycin-cultured islets was similar to that of control islets in the presence of

Table 2 Effect of antimycin A on glucose oxidation and glucose utilization. Islets were cultured without rapamycin for 24 h. After cultured islets were preincubated with 2·8 mM glucose for 30 min, they were incubated with 2·8 and 16·7 mM glucose for 90 min with or without 100 nM antimycin A. Values represent mean±S.E.M. of nine (glucose oxidation) and five (glucose utilization) determinations

	Control	100 nM antimycin A
Glucose oxidation		
2·8 mM glucose	9·8±0·5	9·8±0·7
16·7 mM glucose	42·1±1·8	32·8±1·8*
Glucose utilization		
2·8 mM glucose	32·9±3·3	31·1±2·4
16·7 mM glucose	97·9±5·6	76·7±6·5†

*$P<0·01$ versus control without antimycin A. †$P<0·05$ versus control without antimycin A.

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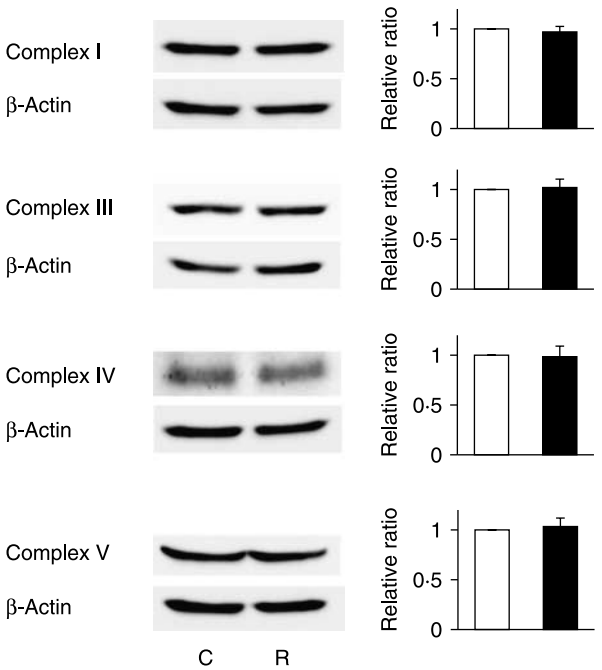

Figure 4 Immunoblots of complex I, III, VI, and V of mitochondrial respiratory chain proteins using lysates of whole-rat pancreatic islets. Islets were cultured with or without 30 nM rapamycin for 24 h. C, control; R, rapamycin. Quantification data from several independent experiments (I, $n=4$; III, $n=4$; VI, $n=3$; V, $n=4$) are also indicated. Open bar, control; closed bar, rapamycin. Data are expressed relative to control (without rapamycin) values corrected by β-actin level to eliminate influence of subtle difference in amount of loaded protein (means ± S.E.M).

succinate plus rotenone, TMPD plus ascorbate, and glycerol-3-phosphate. ATP production by mitochondria from rapamycin-cultured islets in the presence of pyruvate plus malate was decreased compared with that from control islets.

α-Keto-β-methyl-*n*-valeric acid (KMV), a specific competitive inhibitor of KGDH, dose dependently suppressed mitochondrial ATP production in the presence of malate and pyruvate (Fig. 5).

Effect of rapamycin on activities of mitochondrial enzymes

Enzyme activities in the Krebs cycle including PDH, NAD-ICDH, aconitase, and MDH were not affected, but KGDH activity was reduced by rapamycin treatment (Table 4).

Discussion

In the present study, we show that rapamycin suppresses high glucose-induced insulin secretion from pancreatic islets by reducing mitochondrial ATP production through suppression of carbohydrate metabolism in the Krebs cycle, together with reduced KGDH activity. Thus, dysfunction in mitochondrial ATP production may be derived not from alteration in protein expression and dysfunction of the respiratory chain but from decreased KGDH activity that limits the velocity of carbohydrate metabolism in the Krebs cycle.

Rapamycin significantly decreased glucose-induced insulin release after 1 to several days exposure, as found in previous studies using rat islets (Bell *et al.* 2003) and mice islets (Zhang *et al.* 2006). In the present study, exposure to 30 nM rapamycin for 24 h reduced glucose-induced insulin release without affecting insulin and DNA content, which indicates that reduced insulin release by rapamycin is not necessarily derived from reduced β-cell mass, while rapamycin above 10 nM was found to increase apoptosis in MIN-6 cells in a previous study (Bell *et al.* 2003). To investigate the mechanism of reduced insulin release by rapamycin independent of reduced insulin and DNA content, we used 30 nM rapamycin-treated islets. The recommended trough concentrations of rapamycin in blood are 5–15 ng/ml (or 5·5–15·9 nM) in islet transplantation (Shapiro *et al.* 2000) and renal transplantation (Teutonico *et al.* 2005). Accordingly, the concentration used in our experiments was two to six times clinically used trough concentrations.

In pancreatic β-cells, intracellular ATP originated mainly from mitochondria is one of the most important regulators of insulin secretion (Maechler & Wollheim 2001). Glucose entry into the β-cells accelerates glycolysis and mitochondrial carbohydrate metabolism that increases ATP content and ATP/ADP ratio, which closes the ATP-sensitive K^+ channels (K_{ATP} channel). The decrease in K^+ conductance depolarizes the membrane and opens the voltage-dependent Ca^{2+} channels (VDCCs). Increased Ca^{2+} influx through VDCCs increases the intracellular Ca^{2+} concentration to a level that triggers

Table 3 ATP production by mitochondria from control and rapamycin (Rp)-cultured islets. Islets were cultured with or without 30 nM Rp for 24 h. Mitochondrial suspension was obtained from control and Rp-cultured islets. Mitochondrial ATP production is indicated as the ratio to ATP production from adenylate kinase, which was determined from the same sample in parallel incubation. Values represent mean ± S.E.M. of five (A) and three (B) determinations

	Mitochondrial ATP production	
Experimental conditions	Control islets	Rp-cultured islets
A		
1 mM succinate + 1 μM rotenone	6·40 ± 0·32	6·47 ± 0·44
1 mM pyruvate + 1 mM malate	3·22 ± 0·10	2·69 ± 0·11*
0·5 mM TMPD + 2 mM ascorbate	8·74 ± 1·44	8·70 ± 1·34
1 mM glycerol-3-phosphate	0·66 ± 0·13	0·55 ± 0·08
1 mM succinate + 1 μM antimycin A	0·02 ± 0·00	0·02 ± 0·00
B		
1 mM succinate	7·13 ± 0·12	ND
1 mM succinate + 1 μM rotenone	6·39 ± 0·06	ND
1 mM succinate + 1 μM antimycin A	0·02 ± 0·01	ND
1 mM succinate + 1 μM rotenone + 1 μM antimycin A	0·03 ± 0·00	ND

*$P<0·05$ versus corresponding control cultured without Rp. TMPD, tetramethyl-*p*-phenyldiamine. ND, not determined.

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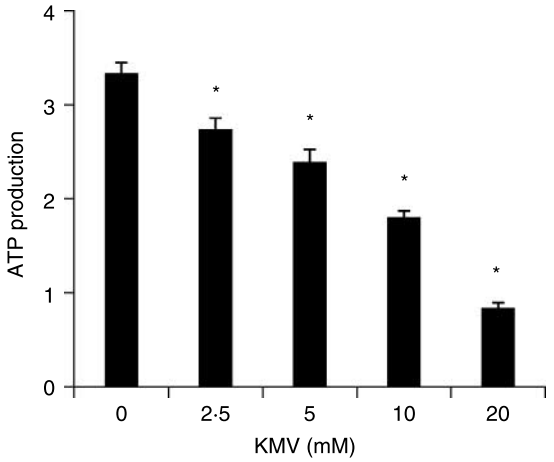

Figure 5 Effect of α-keto-β-methyl-*n*-valeric acid (KMV) on mitochondrial ATP production. Islets were cultured for 24 h. Mitochondrial suspension was obtained from cultured islets. Mitochondrial ATP production in the presence of 1 mM pyruvate and 1 mM malate with various concentrations of KMV is indicated as the ratio to ATP production from adenylate kinase, which was determined from the same sample in parallel incubation. Values represent mean±S.E.M. of six determinations. *$P<0{\cdot}01$ versus control without KMV.

exocytosis of the insulin granules. Moreover, ATP directly affects the exocytotic system and enhances insulin release in experiments using single β-cells (Rorsman 1997, Takahashi *et al.* 1999) and permeabilized islets (Fujimoto *et al.* 2002). Thus, a lower ATP level in the presence of high glucose plays a major role in the attenuation of insulin secretion from rapamycin-treated islets in response to high glucose.

In pancreatic islets, KIC is oxidized, enhancing ATP production and triggering insulin release (Malaisse *et al.* 1981), and two distinct mechanisms of KIC-induced insulin release are proposed. In one, KIC, which is converted to acetyl CoA via a branched chain α-keto acid dehydrogenase (BCKDH) -dependent pathway, enters into the Krebs cycle and is oxidized (Lenzen *et al.* 1982, 1985). In the other, KIC together with endogenous glutamate is converted to α-ketoglutarate via glutamate-keto acid transaminase (GKAT), which enters into the Krebs cycle and is oxidized (Gao *et al.* 2003). Because both BCKDH and GKAT are mitochondrial enzymes, KIC might well be metabolized within mitochondria without affecting cytosolic glycolysis, which is compatible with the results showing that inhibition of glycolysis by glucokinase inhibitor and glyceraldehyde-3-phosphate dehydrogenase inhibitor decreased glucose-induced insulin release, but did not affect KIC-induced insulin release (Radu *et al.* 2005). Reduced KIC-induced insulin release by rapamycin suggests that the decreased glucose metabolism may be derived from reduced mitochondrial carbohydrate metabolism.

Table 4 Effect of rapamycin (Rp) on enzyme activities in the Krebs cycle. Islets were cultured with or without 30 nM Rp for 24 h. Enzyme activities were measured using homogenates of mitochondrial fraction obtained from control and Rp-cultured islets. Values represent mean±S.E.M. of five determinations

	Enzyme activities (nmol/mg mitochondrial protein per min)	
	Control islets	Rp-cultured islets
PDH	10·64±0·38	10·98±0·38
Aconitase	10·83±0·78	9·77±0·78
NAD-ICDH	11·50±0·28	10·99±0·30
KGDH	13·82±0·84	10·08±0·82*
MDH	1116±37	1127±37

*$P<0{\cdot}05$ versus corresponding control cultured without Rp. PDH, pyruvate dehydrogenase; NAD-ICDH, NAD^+ linked-isocitrate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase.

Because rapamycin reduced glucose utilization in the presence of high glucose, which reflects the velocity of glycolysis (Meglasson & Matschinsky 1986), the activity of glucokinase, a rate-limiting enzyme in glycolysis (Matschinsky 1996), was examined. Since rapamycin treatment did not affect glucokinase activity in islets, the primary cause of reduced glucose oxidation by the treatment is not likely to be reduced the velocity of glycolysis. Indeed, in islets, glucose utilization is also reduced when glucose oxidation is decreased by respiratory chain inhibitors including site III inhibitor in our results and site VI inhibitor (Sener *et al.* 2007), suggesting that reduced glucose oxidation may decrease glucose utilization. Since glucokinase is a unique hexokinase, which lacks product inhibition (Matschinsky 2002), accumulation of glucose-6-phosphate by mitochondrial metabolic inhibition may not participate in glycolysis inhibition. Moreover, since K_m of glucokinase for ATP is about 0·5 mM, which is less than the estimated cytosolic ATP concentration with a basal level of glucose in β-cells (about 3 mM; Meglasson & Matschinsky 1984), a decrease in the cytosolic ATP concentration by mitochondrial metabolic inhibition may have little effect on velocity of glycolysis.

Mitochondrial ATP production is driven by the H^+ gradient across the mitochondrial membrane generated by transport of high-energy electrons in the respiratory chain. These electrons are derived from NADH and $FADH_2$ derived from the Krebs cycle in the matrix and/or transferred from the cytosol by the shuttle system. To find the defective site in mitochondrial carbohydrate metabolism in rapamycin-cultured islets, mitochondrial ATP production was examined in the presence of various substrates and inhibitors. As ATP production in the presence of glycerol phosphate was not affected, reduced function of the glycerol phosphate shuttle, which is observed in diabetic islets (Östenson *et al.* 1993), may not participate in the reduction of ATP production by rapamycin treatment. In the presence of rotenone, a complex I inhibitor, and succinate, which renders electrons indirectly to complex I via the Krebs cycle and directly to complex II, electrons are rendered to the respiratory chain via $FADH_2$ at complex II and not at complex I via NADH, which is derived from metabolism in the Krebs cycle. TMPD is an artificial

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electron donor that can transfer electrons to cytochrome *c*. TMPD reduced by ascorbate renders electrons to cytochrome *c*, which transfers electrons to complex IV. The fact that ATP production in the presence of succinate plus rotenone and in the presence of TMPD plus ascorbate is similar in the two groups of mitochondria indicates that the respiratory chain downstream of complex II is not affected by chronic exposure to rapamycin. Moreover, immunoblotting revealed that expressions of respiratory chain proteins including complex I, III, IV, and V were not affected by rapamycin treatment. Antibodies used in the present study were raised against 39 kDa subunit in complex I, core II in complex III, subunit I in complex IV, and subunit α in complex V. In these subunits, subunit I in complex IV is derived from an mtDNA-encoded gene; the others are from nuclear genes (Hunte 2001, Richter & Ludwig 2003, Scheffler 2008, Zickermann *et al.* 2009), indicating that rapamycin does not affect the expression of respiratory proteins derived from mtDNA or nuclear genes in islets. Considered together, these results do not support the notion that rapamycin reduces ATP production by reducing activity of the respiratory chain. Because the decrease in ATP production was found in the presence of substrates that are metabolizable in the Krebs cycle by rapamycin treatment, the reduction in ATP production may be attributable to reduced carbohydrate metabolism in the Krebs cycle.

Glucose oxidation reflects the velocity of carbohydrate metabolism in the Krebs cycle in which CO_2 is released in the reaction mediated by dehydrogenases. To clarify the link between reduced mitochondrial ATP production in the presence of substrates metabolizable in the Krebs cycle and reduced glucose oxidation by rapamycin, recovery of insulin release and ATP content in the presence of high glucose by ROS scavengers and activity of enzymes in the Krebs cycle were examined. Ouabain-induced endogenous ROS suppresses mitochondrial metabolism in the Krebs cycle, subsequently reducing ATP production, and reduces glucose-induced insulin release and ATP levels in the presence of high glucose, which is recovered by the suppression of endogenous ROS production and by ROS scavenge (Kajikawa *et al.* 2002, Kominato *et al.* 2008). High glucose raises ROS level in β-cells (Bindokas *et al.* 2003, Sakai *et al.* 2003), which is also found in our previous study (Kominato *et al.* 2008). However, our previous study shows that ROS scavenging does not affect glucose-induced insulin secretion from control islets, but increases that from GK-diabetic islets. A more profound increase in high glucose-induced ROS was observed in diabetic islets compared with control islets. These results suggest that a physiological level of ROS increase by glucose does not impair stimulus-secretion coupling, while a pathophysiological increase in ROS impairs stimulus-secretion coupling. Administration of H_2O_2, the most abundant ROS, to mitochondria reduced the activity of Krebs cycle enzymes including aconitase, KGDH, and succinate dehydrogenase (Tretter & Adam-Vizi 2000, Nulton-Persson & Szweda 2001). Because α-tocopherol is a lipid-soluble antioxidant, it is often used as membrane-permeable ROS scavenger. α-tocopherol reduces ROS production in various kinds of cells (Saito *et al.* 2003, Brookheart *et al.* 2009, Yang *et al.* 2009) including β-cells (Kajikawa *et al.* 2002). As ascorbate is water soluble, it is not necessarily membrane permeable. However, it is useful to prevent oxidization of α-tocopherol in the medium and to maintain the ROS-scavenging effect of α-tocopherol. Because insulin release and ATP content in the presence of high glucose in rapamycin-treated islets were not increased by the addition of α-tocopherol plus ascorbate, overproduction of endogenous ROS seems not to participate in reduced mitochondrial carbohydrate metabolism due to rapamycin treatment.

Impaired metabolism-secretion coupling in β-cells due to reduced activity of enzymes in the Krebs cycle has been reported. Exposure to fatty acids for 48 h inhibits glucose-induced insulin secretion from islets with decreased activity in PDH (Zhou & Grill 1995). Interleukin-1β-induced nitric oxide production leads to inhibition of glucose-induced insulin secretion together with reduced aconitase activity (Welsh *et al.* 1991). In the present study, activity of KGDH was decreased by rapamycin treatment. The reaction catalyzed by KGDH is one of the slowest steps in the Krebs cycle, and thus can be the rate-limiting step in islets (Ashcroft 1981) and in other tissues (Tretter & Adam-Vizi 2000, Nulton-Persson & Szweda 2001). Inhibition of KGDH alters mitochondrial function in N2a neuroblastoma cells (Huang *et al.* 2003). These findings suggest that decreased activity of KGDH might reduce mitochondrial ATP production and result in decreased glucose-induced insulin secretion from rapamycin-treated islets. To investigate this, suppression of mitochondrial ATP production by inhibition of KGDH was examined using KMV, a specific competitive inhibitor of KGDH (Huang *et al.* 2003). KMV dose dependently suppressed mitochondrial ATP production in the presence of malate and pyruvate. This dose dependency of KMV on mitochondrial ATP production is consistent with the dose-dependent effect of KMV on KGDH activities previously described (Huang *et al.* 2003). These results indicate that reaction at KGDH may limit the velocity of carbohydrate metabolism in the Krebs cycle and thus mitochondrial ATP production, which is consistent with the result that KGDH limits the amount of NADH available for the respiratory chain (Tretter & Adam-Vizi 2000). These results support the notion that a slight alteration in KGDH activity may affect mitochondrial ATP production.

While rapamycin shares with tacrolimus a similar molecular structure and binding ability to FK-binding protein 12 (FKBP12), the FKBP12–rapamycin complex has no effect on calcineurin, a phosphatase that is known to be inhibited by the FKBP12–tacrolimus complex (Saunders *et al.* 2001). This is consistent with our finding in the present study that rapamycin had no effect on glucokinase activity, but tacrolimus suppresses glucokinase activity in islets (Radu *et al.* 2005). MTOR has an FKBP12–rapamycin binding domain to which phosphatidic acid (PA) can also bind.

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MTOR expresses biological effects by forming two types of complexes, MTORC1 and MTORC2, which includes MTOR and PA commonly and Raptor and Rictor respectively. FKBP12–rapamycin is believed to inhibit MTOR signaling by preventing the interaction between MTOR and PA and thus forming MTOR complexes (Foster & Toschi 2009). Since low concentrations of rapamycin (0·5–100 nM) target MTORC1 and higher concentrations of rapamycin (0·2–20 μM) target MTORC2 (Foster & Toschi 2009), our result may be derived from inhibition of signaling mediated by MTORC1.

Recently, it has been revealed that MTOR is a nutrient sensor that balances energy metabolism by transcriptional control of mitochondrial oxidative function using peroxisome proliferator-activated receptor γ coactivator-1α in skeletal muscle cells (Cunningham *et al.* 2007). Further investigation of suppression of KGDH activity by rapamycin is required to clarify adaptation of mitochondrial oxidative function and insulin secretion according to nutrient supply.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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