Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity

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Tacrolimus is widely used for immunosuppression in the treatment of organ transplantation and rejection. The main side effects are hyperglycemia due to reduced insulin secretion. Tacrolimus markedly reduces the number of endocrine secretory granules in human pancreatic β-cells, which correlates with the blood concentration of the agent and is reversible by reduction of the dose. The agent decreases insulin release, insulin content, and insulin mRNA concentration dependently in experiments using cell lines, rat islets, and human islets. Tacrolimus also suppresses insulin gene expression, which is induced by intracellular Ca2+ elevation via the calcineurin/NFAT pathway. Thus reduced insulin content due to reduced insulin synthesis plays an important role in the inhibitory effect of tacrolimus on insulin release.

The mechanism of glucose-stimulated insulin release from pancreatic β-cells has been well documented. Glucose stimulates insulin secretion by both triggering and amplifying signals in pancreatic β-cells. The triggering pathway includes entry of glucose into β-cells, acceleration of glycolysis in cytosol and glucose oxidation in mitochondria, an increase in ATP and ADP ratio, closure of ATP-sensitive K+ (KATP) channels, membrane depolarization, opening of voltage-dependent Ca2+ channels (VDCCs), an increase in Ca2+ influx through VDCCs, raised intracellular Ca2+ concentration ([Ca2+]i), and exocytosis of insulin granules. It has been reported that glucose also enhances insulin secretion via KATP channel independently. The KATP channel-independent amplifying action of glucose has been confirmed by treatment of β-cells with diazoxide, which prevents KATP channels from closing, and with a depolarizing concentration of extracellular K+ that restores Ca2+ influx. Because glucose does not increase [Ca2+]i but nevertheless augments insulin release under these conditions, glucose may well exert its effects by increasing Ca2+ efficacy in stimulation-secretion coupling, due at least in part to the direct effect of increased ATP derived from glucose metabolism on exocytosis. Thus glucose metabolism in β-cells plays a crucial role in glucose-induced insulin release. However, the effect of tacrolimus on glucose-induced insulin release and glucose metabolism has not been examined in detail.

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We have investigated the effects of chronic exposure to tacrolimus on insulin release by functional analysis of the metabolic responses of tacrolimus-treated islets.

**MATERIALS AND METHODS**

**Materials.** Tacrolimus (FK-506) was donated by Fujisawa Pharmaceutical (Tokyo, Japan). RPMI 1640 and glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Sigma (St. Louis, MO). Luciferin-luciferase was obtained from Turner Designs (Sunnyvale, CA). H2O and [5-^3^H]glucose were obtained from Amersham (Buckinghamshire, UK). All other agents were obtained from Nacalai Tesque (Kyoto, Japan).

**Animals.** Male Wistar rats weighing 180–230 g were obtained from Shimizu (Kyoto, Japan). The animals were fed standard lab chow ad libitum and allowed free access to water in an air-conditioned room with a 12:12-h light-dark cycle until the experiments. All experiments were carried out with rats aged 8–12 wk. 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 described previously (8). Isolated islets were cultured for 24 h in RPMI 1640 medium containing 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 5.5 mM glucose with or without tacrolimus at 37°C in humidified air containing 5% CO2.

**Measurement of insulin release from isolated rat pancreatic islets.** Insulin release from intact islets was monitored using either batch incubation or a perfusion system described previously, using Kreh-Ringer bicarbonate buffer (KRBB) supplemented with 0.2% bovine serum albumin (BSA; fraction V) and 10 mM HEPES, adjusted to pH 7.4 (KRBB medium) (8).

For static incubation experiments, cultured islets were preincubated at 37°C for 30 min in KRBB medium supplemented with 2.8 mM glucose. Groups of five islets were then batch incubated for 30 min in 0.7 ml of KRBB medium with test materials. At the end of the incubation period, the islets were pelleted by centrifugation, and aliquots of the buffer were sampled.

For perfusion experiments, groups of 20 cultured islets were placed in each of the parallel chambers of a perfusion apparatus and perfused with KRBB medium at a rate of 0.7 ml/min at 37°C. The medium was continuously gassed with 95% O2-5% CO2. The islets were perfused for 30 min to establish a stable insulin secretory rate at a basal level of glucose and then exposed to the stimulating levels of glucose.

The amount of immunoreactive insulin was determined by RIA with rat insulin as standard.

**Measurement of insulin and DNA content.** Insulin contents and DNA contents of islets were measured as previously described (10). Briefly, after an aliquot of incubation medium for insulin release assay was taken, the islets remaining were lysed. Insulin content was determined by RIA using diluted samples. DNA content was determined by fluorometric assay using bisbenzimidazole (Hoechst 33258) with a 340-nm excitation and a 450-nm emission.

**Measurement of NAD(P)H fluorescence.** Cultured islets without dye were immediately placed in a heat-controlled chamber on the stage of an inverted microscope kept at 36 ± 1°C and superfused with KRBB medium with 2.8 mM glucose for 30 min. The islets were excited successively at 360 nm, and the fluorescence emitted at 470 nm (13) was captured every 20 s by a charge-coupled device camera (Micro Max 5-MHz System; Roper Industries, Trenton, NJ). Changes in NAD(P)H fluorescence signal were expressed as percent control values by dividing the signal at a given time by the average signal at 2.8 mM glucose during the last 5 min before stimulation. Images of NAD(P)H were analyzed with the MetaFluor image analyzing system (Universal Imaging, West Chester, PA).

**Measurement of ATP content.** ATP contents were determined as previously described, with a slight modification (10). Briefly, after groups of cultured islets were preincubated at 2.8 mM glucose for 30 min, groups of 15 islets were batch incubated in 0.5 ml of KRBB medium containing 2.8 or 16.7 mM glucose at 37°C for 30 min. Incubation was stopped by the addition of 0.1 ml of 2 M HClO4. The tubes were immediately mixed with vortex and sonicated in ice-cold water. They 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 Na2CO3. The ATP concentration was measured by adding 0.2 ml of luciferin-luciferase solution to a fraction sample (0.1 ml) in a bioluminometer (Luminometer model 20e; Turner Designs). To draw a standard curve, blanks and ATP standards were run through the entire procedure, including the extraction steps.

**Measurement of glucose utilization.** Glucose utilization was measured, using a previously described method (2) with a slight modification. Cultured islets were preincubated in KRBB medium with 2.8 mM glucose at 37°C for 30 min. Batches of 30 islets for each condition were incubated at 37°C for 90 min in 150 μl of medium containing 1.5 μCi of [5-^3^H]glucose. Aliquots of the incubation medium (100 μl) and 20 μl of 1 M HCl were transferred into small tubes and placed into a glass vial containing 0.5 ml of H2O. The capped vials were incubated overnight at 37°C to vaporize H2O from the solution. The inner tube was then lifted out, and the disintegrations per minute of water-melting H2O in the vial were counted. In parallel incubation, the recovery ratio of H2O was measured using 3H2O. After subtracting blank disintegrations per minute from sample disintegrations per minute, glucose utilization was calculated using the disintegrations per minute, specific radioactivity of [5-^3^H]glucose, and recovery ratio of H2O.

**Measurement of glucokinase and hexokinase activity.** Glucokinase activity was measured by a fluorometric assay according to a method reported previously (25, 38). After cultured islets were preincubated with KRBB medium with 2.8 mM glucose, 100 islets were homogenized in 250 μl of solution containing 20 mM KH2PO4, 100 mM KCl, 1 mM MgCl2, 1 mM EDTA, 5% glycerol, and 1 mM DTT (pH 7 by KOH), and the supernatants (islet extracts) were obtained from the homogenates by centrifugation (10,000 g, 15 min) at 4°C. The glucokinase phosphorylation rate was estimated as the increase in NADH through the following reaction: glucose-6-phosphate + NAD → 6-phosphoglucono-6-lactone + NADH by NAD-dependent G6PDH. The enzyme reaction was performed using 25 μl of islet extracts in a 160-μl solution consisting of 50 mM HEPES (pH 7.4 by NaOH), 100 mM KCl, 8 mM MgCl2, 0.5 mM NAD, 5 mM ATP, 1 mM DTT, and 1 U/ml G-6-PDH supplemented with two concentrations (50 mM and 0.5 mM) of glucose at 37°C for 1 h; it was stopped by adding 290 μl of stopping solution (300 mM Na2HPO4. 0.46 mM SDS, pH 8.0). NADH concentration was measured by fluorometry (Shimazu RF-5000, Kyoto, Japan) at a 340-nm excitation and a 450-nm emission. Blanks in the absence of ATP were incubated in a parallel experiment and were subtracted from the total fluorescence of the corresponding complete reaction mixtures. Glucokinase activity was determined by subtracting hexokinase activity measured at 0.5 mM glucose from the activity measured at 50 mM glucose.

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance was evaluated by unpaired Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

**Effect of chronic exposure to tacrolimus on glucose-induced insulin release, insulin content, and DNA content in islets.** Chronic (24 h) exposure to tacrolimus (3–30 nM) concentrations dependently suppressed 16.7 mM glucose-induced insulin release but did not affect basal insulin release in the presence of 2.8 mM glucose (Table 1). Insulin content was significantly reduced by 24-h exposure to 10 and 30 nM tacrolimus (Table 2). However, 24-h exposure to 3 nM tacrolimus...
Table 1. Concentration-dependent effect of chronic exposure to tacrolimus on glucose-induced insulin release from pancreatic islets

<table>
<thead>
<tr>
<th>Experimental Condition During Culture</th>
<th>Insulin Release, ng/islet (-30 \text{ min}^{-1})</th>
<th>Glucose Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.02</td>
<td>Basal (2.8 mM glucose)</td>
</tr>
<tr>
<td>3 nM Tacrolimus</td>
<td>0.26±0.03</td>
<td>Stimulated (16.7 mM glucose)</td>
</tr>
<tr>
<td>10 nM Tacrolimus</td>
<td>0.24±0.02</td>
<td></td>
</tr>
<tr>
<td>30 nM Tacrolimus</td>
<td>0.21±0.02</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SE of 16 determinations from several experiments. Islets were cultured under the conditions indicated for 24 h. After cultured islets were preincubated with 2.8 mM glucose for 30 min, insulin release in the presence of 2.8 and 16.7 mM glucose for 30 min was determined. *P < 0.01 vs. control (cultured without tacrolimus).

Table 2. Effect of chronic exposure to tacrolimus on insulin content and DNA content

<table>
<thead>
<tr>
<th>Experimental Condition During Culture</th>
<th>Insulin Content, ng/islet</th>
<th>DNA Content, ng/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.0±0.7</td>
<td>19.3±0.7</td>
</tr>
<tr>
<td>3 nM Tacrolimus</td>
<td>26.2±1.0</td>
<td>21.1±0.8</td>
</tr>
<tr>
<td>10 nM Tacrolimus</td>
<td>22.4±1.1*</td>
<td>20.1±1.0</td>
</tr>
<tr>
<td>30 nM Tacrolimus</td>
<td>20.4±0.8*</td>
<td>19.9±1.0</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 16 determinations from several experiments. The end of the experiments indicated in Table 1, insulin content and DNA content were determined, using islets in a randomly selected half of the batches. *P < 0.01 vs. control (cultured without tacrolimus).

Table 3. Reversible effect of tacrolimus on glucose-induced insulin release from islets

<table>
<thead>
<tr>
<th>Experimental Condition During Culture</th>
<th>Insulin Release, ng/islet (-30 \text{ min}^{-1})</th>
<th>Glucose Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.02</td>
<td>Basal (2.8 mM glucose)</td>
</tr>
<tr>
<td>24-h Tacrolimus</td>
<td>0.26±0.02</td>
<td>Stimulated (16.7 mM glucose)</td>
</tr>
<tr>
<td>12-h Tacrolimus + 12-h no tacrolimus</td>
<td>0.24±0.01</td>
<td></td>
</tr>
<tr>
<td>12-h Tacrolimus + 12-h no tacrolimus</td>
<td>0.28±0.03</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SE of 8 determinations from several experiments. Two groups of islets were cultured with or without 3 nM tacrolimus for 24 h (control and 24-h tacrolimus group). One group of islets was cultured without tacrolimus for the first 12 h and with 30 nM tacrolimus for the second 12 h (12-h no tacrolimus + 12-h tacrolimus group). Another group of islets was cultured with 30 nM tacrolimus for the first 12 h and without tacrolimus for the second 12 h (12-h tacrolimus + 12-h no tacrolimus group). After cultured islets were preincubated with 2.8 mM glucose for 30 min, insulin release in the presence of 2.8 and 16.7 mM glucose for 30 min was determined. *P < 0.01 vs. control (24-h no tacrolimus). †P < 0.01 vs. 24-h tacrolimus.
high glucose in both groups of islets and reached a plateau within 10 min after exposure (Fig. 2A). A significant attenuation of NAD(P)H fluorescence was observed from 10 min in tacrolimus-treated islets (at 10 min: tacrolimus 124.9 ± 1.3, n = 11, vs. control 129.3 ± 1.1%, n = 12, P < 0.05; Fig. 2A). Mean NAD(P)H fluorescence during the first 10 min after high-glucose exposure was not affected, whereas that during the second and third 10-min period was suppressed in tacrolimus-treated islets [mean NAD(P)H fluorescence from 20 to 30 min: tacrolimus 124.5 ± 1.2, n = 11, vs. control 133.9 ± 1.4%, n = 12, P < 0.01; Fig. 2B].

Effect of tacrolimus on ATP content. ATP content was greater in islets incubated with 16.7 mM glucose than in islets incubated with 2.8 mM glucose in both control (2.8 mM glucose, 5.22 ± 0.51, vs. 16.7 mM glucose, 9.69 ± 0.99 pmol/islet, n = 10, P < 0.01) and tacrolimus-treated islets (2.8 mM glucose, 4.75 ± 0.50, vs. 16.7 mM glucose, 6.52 ± 0.40 pmol/islet, n = 10, P < 0.05). ATP content in the presence of 16.7 mM glucose was significantly reduced in tacrolimus-treated islets (P < 0.01) but in the presence of 2.8 mM glucose was not affected by tacrolimus (Fig. 3A).

Effect of tacrolimus on glucose utilization. Glucose utilization was greater in islets incubated with 16.7 mM glucose than in islets incubated with 2.8 mM glucose in both control (2.8 mM glucose, 35.1 ± 6.1, vs. 16.7 mM glucose, 103.8 ± 6.9 pmol/islet⁻¹-h⁻¹, n = 9, P < 0.01) and tacrolimus-treated islets (2.8 mM glucose, 24.6 ± 3.7, vs. 16.7 mM glucose, 73.4 ± 5.1 pmol/islet⁻¹-h⁻¹, n = 9, P < 0.01). Glucose utilization in the presence of 16.7 mM glucose was significantly reduced in tacrolimus-treated islets (P < 0.01) but in the presence of 2.8 mM glucose was not affected by tacrolimus (Fig. 3B).

Effect of tacrolimus on glucokinase and hexokinase activity. Glucokinase activity was significantly decreased by the tacrolimus treatment (tacrolimus 49.9 ± 3.4 vs. control 65.3 ± 3.4 pmol-islet⁻¹-hour⁻¹, n = 10, P < 0.01), whereas hexokinase activity was not affected (tacrolimus 35.5 ± 2.8 vs. control 34.1 ± 2.1 pmol-islet⁻¹-hour⁻¹, n = 10, not significant; Fig. 3C).

**DISCUSSION**

In the present study, we show that a low concentration of tacrolimus suppresses high glucose-induced insulin secretion from pancreatic islets without affecting insulin content. This inhibitory effect of tacrolimus is the result of reduced ATP production and glycolysis due to decreased glucokinase activity.

We found that 3 nM tacrolimus, a lower concentration than used in previous reports (31, 32, 41), significantly decreased glucose-induced insulin release after 24-h exposure without affecting insulin content, which indicates that reduced insulin release by tacrolimus is not necessarily derived from reduced insulin content. To investigate the mechanism of reduced insulin release by tacrolimus independent of reduced insulin content, we used 3 nM tacrolimus-treated islets. Recommended trough concentrations of tacrolimus in blood are 3–6 ng/ml (3.6–7.2 nM) and 5–15 ng/ml (6.1–18.2 nM) in islet transplantation (36) and in liver transplantation (3), respectively.
chondrial membrane generated by transport of high-energy elec-
trons in the respiratory chain. These electrons are derived from
NADH and FADH₂, which are derived from the TCA cycle in the
matrix and/or transferred from the cytosol by the shuttle system.
A reduced supply of substrates to mitochondria results in a
decreased H⁺ gradient across the mitochondrial membrane. Glu-
cose utilization reflects the velocity of glycolysis (24), and
NAD(P)H autofluorescence dominantly reflects the redox state of
mitochondria (29). Because both glucose utilization and
NAD(P)H fluorescence in the presence of high glucose were
decreased in tacrolimus-treated islets, the reduced ATP content in
these islets may well be attributable to a decreased supply of
reduced equivalents to mitochondria.

In pancreatic islets, KIC is oxidized, enhancing ATP pro-
duction and triggering insulin release (21). However, the mecha-
nism of KIC-induced insulin release is not fully understood,
and two distinct mechanisms are proposed. First, KIC, which is
converted to acetyl-CoA via a branched-chain α-ketoacid de-
hydrogenase (BCKDH)-dependent pathway, enters into the
TCA cycle and is oxidized (18, 19). Second, KIC, together
with endogenous glutamate, is converted to α-ketoglutarate via
glutamate-ketoacid transaminase (GKAT), which enters into
the TCA cycle and is oxidized (11). Because both BCKDH and
GKAT are mitochondrial enzymes, KIC should be metabolized
within mitochondria without affecting cytosolic glycolysis. Our
results show that mannoheptulose, a glucokinase inhibitor
(39), and iodoacetate, a glyceraldehyde-3-phosphate dehydro-
genase inhibitor (35), both of which inhibit glycolysis, de-
creased glucose-induced insulin release but did not affect
KIC-induced insulin release. These results are compatible with
the notion that KIC is metabolized within mitochondria with-
out affecting cytosolic glycolysis. Accordingly, KIC-induced
insulin release was examined to clarify the effect of tacrolimus
on mitochondrial metabolism independent of glycolysis. Be-
cause tacrolimus reduced glucose-induced insulin release but
did not affect KIC-induced insulin release, the decreased glu-
cose metabolism should be derived from reduced glycolysis.

Glucokinase is a rate-limiting enzyme in glycolysis and
serves as glucose sensor in pancreatic β-cells (22). Regulation
of the enzyme has a central impact on β-cell function, as
demonstrated in studies of glucokinase mutations identified in
patients with maturity onset diabetes of the young (42). The
activity of the enzyme is reduced in tacrolimus-treated islets
and presumably plays a primary role in reducing glucose-
induced insulin release without a decrease in insulin content by
the agent.

Recently, Uchizono et al. (41) reported an effect of chronic
exposure to tacrolimus (mainly 100 nM) on the mechanism of
insulin release under reduced insulin content. Interestingly,
glucose-induced insulin release was found to be more pro-
dfoundly suppressed than mitochondrial fuel-induced insulin
release, a phenomenon clarified by our results. They assert that
tacrolimus impairs glucose-induced insulin release and has
effects distal to the rise in intracellular Ca²⁺, which could be
explained by a reduced ATP level by the agent, as ATP directly
enhances Ca²⁺ efficacy in the exocytotic system of insulin
release (9, 33, 40).

Glucokinase activity in pancreatic β-cells is regulated on
both translational and posttranslational levels, but the mecha-
nism remains largely unknown (23). Further investigation of
suppression of glucokinase activity by tacrolimus may clarify
the regulation of glucokinase activity in pancreatic β-cells.

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**Fig. 3.** Metabolic parameters in control (open bars) and tacrolimus-treated islets (solid bars). Islets were cultured with or without 3 nM tacrolimus for 24 h. Cultured islets were preincubated with 2.8 mM glucose for 30 min. A: ATP content. After preincubation, islets were incubated in the presence of 2.8 or 16.7 mM glucose. Values represent means ± SE of 10 determinations from several experiments. B: glucose utilization. After preincubation, islets were incubated in the presence of 2.8 or 16.7 mM glucose. Values represent means ± SE of 9 determinations from several experiments. C: glucokinase (GK) and hexokinase (HK) activities. Values represent means ± SE of 10 determinations from several experiments. *P < 0.01 vs. corresponding control.

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Accordingly, the concentration used in our experiments is com-
parable to clinically used concentrations.

Chronic exposure to 1 μM tacrolimus for 7 days has been
shown not to alter the extent of islet cell apoptosis or necrosis
(41), suggesting that apoptosis and necrosis in islets are not
increased in our experimental condition. In addition, the inhib-
itory effect of chronic exposure to 3 nM tacrolimus was almost
completely recovered by withdrawal of tacrolimus. For these
reasons, the reduced glucose-induced insulin release from 3
nM tacrolimus-treated islets is unlikely to be derived from
nonspecific irreversible toxic effects of the agent.

In dynamic experiments, both glucose-induced biphasic insulin
release and glucose-induced elevation of NAD(P)H fluores-
cence were reduced by tacrolimus treatment after 10 min of glucose
exposure, suggesting that the reduced insulin release results from
reduced glucose metabolism. Accordingly, ATP, the most im-
portant metabolic signal in insulin release, was investigated. Because
insulin content per islet was not different between control and
tacrolimus-treated islets in the present study, the sizes of the stable
pool of ATP in an islet, which occurs mainly in the insulin
granules, and the diffusible pool, which occurs mainly in the
cytosol (4), were not very different. Therefore, the comparison of
total ATP content is valid. ATP content in the presence of high
glucose was reduced in tacrolimus-treated islets. Mitochondrial
ATP production is driven by the H⁺ gradient across the mito-
chondrial membrane generated by transport of high-energy elec-
tron.
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