Rapamycin Induces Autophagy in Islets: Relevance in Islet Transplantation

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ABSTRACT

Islet transplantation can provide insulin independence in patients with type 1 diabetes mellitus. However, islet allograft recipients exhibit a gradual decline in insulin independence, and only 10% do not require insulin at 5 years. This decline may reflect drug toxicity to islet β cells. Rapamycin, a central immunosuppressant in islet transplantation, is a mammalian target of rapamycin inhibitor that induces autophagy. The relative contributions of autophagy in transplanted islets are poorly understood. Therefore, in the present study we sought to evaluate the effects of rapamycin on islet β cells. Rapamycin treatment of islets resulted in accumulation of membrane-bound light chain 3 (LC3-II) protein, an early marker of autophagy. In addition, rapamycin treatment of isolated islets elicited not only reduction of viability but also downregulation of in vitro potency. To further examine the occurrence of autophagy in rapamycin-treated islets, we used GFP (green fluorescent protein)–LC3 transgenic mice that express a fluorescent autophagosome marker. The GFP-LC3 signals were markedly increased in rapamycin treated islets compared with control islets. In addition, to show improvement by blockade of autophagic signaling, islets were treated with rapamycin in the presence of 3-methyladenine, which inhibits autophagy. Thereafter, both islet viability and islet potency were dramatically improved. The number of GFP-LC3 dots clearly increased after 3-MA treatment. Thus, rapamycin treatment of islets induces autophagy in vitro. This phenomenon may contribute to the progressive graft dysfunction of transplanted islets. Therapeutically targeting this novel signaling may yield significant benefits for long-term islet survival.

Clinical islet transplantation in patients with type 1 diabetes mellitus has recently increased because of the results of the Edmonton protocol, a rapamycin-based, glucocorticoid-free, immunosuppressive regimen that led to insulin independence at 1 year in 90% of treated patients. However, long-term follow-up indicated marked reduction in graft function; only 10% of islet recipients maintained insulin independence at 5 years. While the causes of decline in insulin independence rates seen in
clinical islet transplantation are currently not fully understood, this decline may reflect chronic toxicity of immunosuppressive drugs on islet β cells.

Rapamycin is widely used both as an induction and maintenance immunosuppressant in islet transplantation as part of the original Edmonton protocol. Rapamycin may have deleterious effects on islet β cells. The immunosuppressive mechanism of rapamycin is based on blockade of mammalian target of rapamycin (mTOR), a molecule with a pivotal role in cell cycle progression from late G1 into S phase in response to T-cell growth factor stimulation. The mTOR, which is ubiquitously expressed in various cell types, is a serine/threonine protein kinase that regulates important cellular process including growth, proliferation, motility, survival, protein synthesis, and transcription. Furthermore, mTOR activity inhibits autophagy in cells ranging from yeast to human. Accordingly, the ability of rapamycin to inhibit mTOR activity may induce autophagy.

Autophagy, meaning to eat oneself, is one of the main mechanisms for maintaining cellular homeostasis. Although this pathway is not directly a death pathway, it is a self-cannibalistic pathway. Mediated via lysosomal degradation, autophagy is responsible for destroying cellular proteins and degrading cellular organelles, recycling them to ensure cell survival. Although altered autophagy has been observed in various diseases, including neurodegenerative diseases, cancers, and cardiac myopathies, its role is not known; the crux of the problem is whether the response is cell protective or a mechanism of death.

The relative contributions of autophagy are poorly understood in transplanted islets. The objective of the present study was to evaluate the effects of rapamycin on islet β-cells, including autophagy induction, viability, and insulin secretion, factors that may strongly contribute to progressive dysfunction of transplanted islets.

MATERIALS AND METHODS
Isolation of Pancreatic Islets
Anesthetized male BL6 mice underwent bile duct cannulation with pancreatic inflation using 3 mL of extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution containing 1 mg/mL of collagenase. The inflated pancreas was excised; cleaned of lymph nodes, fat, and bile duct; and digested with collagenase VIII, followed by purification using a discontinuous Ficoll gradient. Isolated islets were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium.

Western Blot Analysis
Western blot analysis was performed to detect the accumulation of LC3-II, an LC3-phosphorylated conjugate, which is an early marker of autophagy. Fresh islets (30 per well) were incubated for 24 hours in culture medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Protein samples from lysed cells underwent electrophoresis with 15% sodium dodecylsulfate–polyacrylamide gel and were transferred to polyvinylidene fluoride membranes. Lysate LC3-II was recognized by immunoblotting with an anti-LC3 monoclonal antibody (MBL International Corp, Woburn, Massachusetts). As the loading control for the samples, we also detected protein expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Protein expression levels of both LC3-II and GAPDH, expressed in arbitrary units, were quantified using an image analyzer (Fluor-Chem; Bio-Rad Laboratories, Inc, Hercules, California). The relative protein expression of LC3-II in islets was normalized to that of GAPDH and expressed as the ratio of LC3-II to GAPDH.

Islet Viability Assay
Thirty cells of fresh mice islets were cultured for 24 hours with complete medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Subsequently, islet viability was determined using the colorimetric methyl tetrazolium salt (MTS) Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin). The colorimetric reagent was added to each well and incubated for 2 hours before absorbance values were read at 490 nm.

Blocking Assay of Autophagic Signaling
To determine whether rapamycin-treated islets recovered their viability, they were assessed using the MTS assay in the absence or presence of 10 mmol/L of 3-methyladenine (3-MA), an inhibitor of class 3 phosphatidylinositol 3-kinase, an inhibitor of autophagy.

Glucose-Stimulated Insulin Release and Stimulation Index
To further determine the in vitro potency of rapamycin-treated islets, static glucose challenge was performed in the absence or presence of 10 mmol/L of 3-MA. After overnight culture, islets were incubated with either 2.8 or 20 mmol/L of glucose in culture medium for 2 hours at 37°C. The collected supernate was stored at −80°C for measurement of insulin with an enzyme-linked immunosorbent assay. Glucose-stimulated insulin release was expressed as the stimulation index, that is, the ratio of insulin release during exposure to high glucose (20 mmol/L) incubation compared with low glucose (2.8 mmol/L) incubation.

Generation of GFP-LC3 Transgenic Mice
For ex vivo studies to monitor autophagy in rapamycin-treated islets, transgenic mice expressing GFP-LC3 under the control of the constitutive CAG (chicken β-actin) promoter were purchased from RIKEN BioResource Center, Wako, Japan. Fresh mouse islets, isolated as described above, were incubated for 24 hours in culture medium in the absence or presence of 1 ng/mL of rapamycin. In addition, rapamycin-treated islets were incubated in the presence of 10 mmol/L of 3-MA. Either untreated control islets, rapamycin-treated islets, or rapamycin plus 3-MA-treated islets were directly observed using a fluorescence microscope (Biozero; Keyence Corp, Osaka, Japan) to detect GFP-LC3 dots.

RESULTS
Endogenous LC3-II Markedly Accumulates in Islets Treated With Rapamycin
Endogenous LC3-II protein was detected in control islets (Table 1). From the results for islets treated with either 1 or 10 ng/mL of rapamycin, the expression level of endogenous LC3-II in 1 ng/mL of rapamycin-treated islets was similar to that in control islets. However, the amount of endogenous LC3-II was doubled in 10 ng/mL of rapamycin-treated islets.
Under blocking conditions of autophagic signaling by 3-MA, there was an approximately 32% reduction in the amount of LC3-II observed in rapamycin-treated islets in the presence of 10 mmol/L of 3-MA as judged by the LC3-II/GAPDH ratio (Table 1).

### Rapamycin Treatment of Islets Results in Reduced Islet Viability

To assess the direct effects of rapamycin on islet viability, we performed the MTS assay. On the basis of treatment with rapamycin, there were approximately 43% and 51% reductions in viability with treatment with 1 and 10 ng/mL of rapamycin, respectively (Table 1). In contrast, the viability of rapamycin-treated islets markedly recovered in the presence of 3-MA. Approximately 69% and 76% islet viability was noted after treatment with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA, and with 10 ng/mL of rapamycin plus 10 mmol/L of 3-MA, respectively (Table 1).

### Rapamycin Strongly Affects In Vitro Islet Function

Islet potency was assessed using a static glucose challenge in vitro. The stimulation index (SI) of untreated control islets was 1.38 ± 0.16 (Fig 1). However, treatment of islets with rapamycin dramatically reduced the SI. The SI was 1.11 ± 0.01 with 1 ng/mL of rapamycin, and no insulin output occurred with 10 ng/mL of rapamycin treatment (Fig 1). In contrast, the SI dramatically improved with the addition of 3-MA. Islets treated with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA, completely recovered compared with untreated control islets (Fig 1). These results indicate that rapamycin treatment of isolated islets elicited not only autophagy induction but also reduced islet viability and potency.

### GFP-LC3 Signal is Strong in Rapamycin-Treated Islets

Autophagy in response to rapamycin treatment was seen on fluorescence photomicrographs of islet samples prepared from GFP-LC3 transgenic mice (Fig 2). In untreated control islets, the GFP-LC3 signal was detected diffusely in islets with few punctuate dots (Fig 2A). After 24 hours of incubation with 1 ng/mL of rapamycin, the number of GFP-LC3 dots markedly increased; most were detected as cup- or ring-shaped structures (Fig 2B). In contrast, the level of GFP-LC3 signals of rapamycin-treated islets in the presence of 10 mmol/L of 3-MA was diffuse and returned to the base level of control islets (Fig 2C).
DISCUSSION

Rapamycin, which is a natural bacterial product that inhibits mTOR by association with an intracellular receptor FKBP12,14 is widely used as the central immunosuppressant in islet transplantation. As is well known, mTOR is a kinase that regulates important cellular processes such as inhibition of autophagy. Our results demonstrate that rapamycin treatment of isolated islets induced autophagy. This phenomenon impaired both islet viability and potency. These deleterious effects of rapamycin on islet β cells were markedly improved by the addition of 3-MA, which is an inhibitor of autophagy. Accordingly, therapeutically targeting this novel pathway may yield significant benefits, preventing the progressive islet graft dysfunction observed in transplant recipients.

REFERENCES


Fig 2. Islet autophagy in response to rapamycin treatment. Islet samples were prepared from GFP-LC3 transgenic mice. A, Untreated control islets. B, Islets treated with 1 ng/mL of rapamycin. C, Islets treated with rapamycin plus 3-MA. Bars indicate 100 μm.