Rapamycin induces autophagy and exacerbates metabolism associated complications in a mouse model of type 1 diabetes
Zhengyu Zhou, Shuyan Wu, Xinyin Li, Zhimou Xue & Jian Tong*
Medical College of Soochow University, Suzhou, P. R. China

Received 5 August 2009; revised 22 September 2009

Type 1 diabetes mellitus (T1DM) is characterized by lack of insulin production as a consequence of massive beta cells destruction. The contributions of autophagy to loss of beta cell mass were not clearly elucidated. Rapamycin is a specific and potent inhibitor of mammalian target of rapamycin (mTOR) and is used as the central immunosuppressant in T1DM patients especially for those who received islet transplantation. In the present study, effects of rapamycin on autophagy of T1DM were investigated in a mouse model treated with multiple low doses of streptozotocin. Rapamycin treatment led to hyperglycemia, weight loss, increased intake of food and drinking water, and islet inflammation in T1DM mice. Pathological changes including autophagy and apoptosis in pancreas, kidney, spleen and thymus, accompanied with an accumulation of LC3-II, Beclin1 and Caspase-3 protein were observed. The results indicate that rapamycin may exacerbate metabolism associated complications by activating autophagy and apoptosis in T1DM.

Keywords: Autophagy, Mouse model, Rapamycin, Type 1 diabetes mellitus

According to WHO report, more than 180 million people worldwide are now suffering from diabetes as a major health threat, and this number is likely to double by 20301. Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results in destruction of insulin-producing beta cells of the pancreas which has been frequently found in children and young people. T1DM patients rely on exogenous insulin for survival, but unfortunately they often develop insulin resistance, chronic vascular and neurological complications after insulin replacement therapy. Islet transplantation provides insulin independence, however the independency decreases gradually over time, and only 10% of the patients may not require insulin in 5 years2. To deal with the problem, studies on T1DM pathogenesis have attracted great medical attention in recent years.

Although the cause of T1DM is still not fully understood, the damage of immune system by environmental and genetic factors has been considered to be involved. Rapamycin, also known as sirolimus, is a macrolide fungicide with potent antimicrobial and antitumor properties. It was found to have immunosuppressive effects, and first tried clinically in 1989 as a treatment for graft-versus-host-rejection (GVHR) after organ transplantation3. Rapamycin can inhibit the clonal expansion of activated lymphocytes by interacting with the mammalian target of rapamycin (mTOR) and inhibiting progression from the G1 to the S phase of the cell cycle4. Nowadays, rapamycin is routinely used as a central immunosuppressant to prevent rejection in organ transplantation, and is likely to be useful in the treatment of autoimmune diseases including T1DM. However, conclusions from previous investigations of rapamycin on the function and survival of beta cells in pancreatic islets from various clinical follow-up studies and animal experiments have often been paradoxical5-9.

Autophagy, meaning to eat itself, is a cellular degradation-recycling system for aggregated proteins and damaged organelles. It has also been considered as an alternative pathway for programmed cell death (type II programmed cell death). Unlike apoptosis, autophagy is a “double-edged sword”. Normal autophagy is beneficial, since it regulates aged protein turnover, eliminates damaged organelles and protects cells during starvation. Nevertheless, autophagy also
may participate in the destruction of cells, as a result of a protracted atrophy of the cytoplasm, beyond a not yet clearly defined point-of-no-return. Dysregulated autophagy is implicated in various diseases including cancers, neurodegeneration, infectious diseases, etc. Autophagy is inhibited by the insulin-amino acid-mTOR signaling pathway. Rapamycin can inhibit mTOR activity and induce autophagy. Data suggest that autophagy is associated with the pathogenesis and prognosis of diabetes and may play a protective or detrimental role depending on the environment. As an activator of autophagy, it is presumed that rapamycin could display effects other than immunosuppression on the progression of diabetes. The present study has been undertaken to demonstrate whether the altered autophagy induced by rapamycin may contribute to cell damage in a mouse model of T1DM.

Materials and Methods

Chemicals—Streptozotocin and rapamycin were purchased from Sigma, St. Louis, MO, USA. Antibodies to LC-3, Beclin 1 and Caspase-3 were obtained from Abgent, USA. Micro BCA Protein Assay Reagent Kit was purchased from Pierce, Rockford, IL, USA. Nitrocellulose membranes were from Bio-Rad, Hercules, CA, USA. Mouse Glycated Hemoglobin A1c, Total Cholesterol and Triglyceride ELISA Kit were all from GBD, Canada.

Animals, induction of diabetes, and experimental design—C57BL/6J male mice, 10 weeks old, were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The experimental mice were given intraperitoneal injections of freshly prepared streptozotocin (STZ, 40 mg/kg in 0.01 M citrate buffer) on 5 consecutive days to induce T1DM model, while the normal control group was injected with the buffer only. Five days after injection, blood was collected from the tip of the tail vein, and non-fasting blood glucose concentrations were measured by glucose oxidase method. Mice with glucose levels above 300 mg/dl were diagnosed as diabetic. The mice were then randomly divided into the following 4 groups of 10 each: Gr.I: nondiabetic, Gr.II: rapamycin-treated nondiabetic, Gr.III: diabetic, and Gr.IV: rapamycin-treated diabetic. Rapamycin (2.0 mg/kg body weight, 3 days/week) was injected intraperitoneally. This dose was two times lower than that used for anti-cancer treatment (4.0 mg/kg body weight). Body weight, food and water intake and tail blood glucose were monitored periodically until 16 days after the last rapamycin administration. The mice were sacrificed by cervical dislocation for the following experiments. Blood was collected and serum was stored at -80°C for subsequent analysis of glycated hemoglobin A1c (HbA1c), total cholesterol and triglyceride levels. They were measured by Enzyme Linked Immunosorbent Assay (ELISA) kits according to the manufacturer’s suggestions. The protocol for this study was approved by the Administration Committee of Laboratory Animal, and the experimental procedure was in line with the Principles of Laboratory Animal Care.

Histology analysis of pancreas and kidney—The pancreas and kidney were dissected out and fixed in 10% buffered formalin solution, then embedded in paraffin, cut, and stained with haematoxylin-eosin. Histopathologic evaluation was performed in a blinded fashion. The severity of insulitis was determined by the area size of lymphocytic infiltration based on the scoring system. Score 0 indicates islet integrity without lymphocytic infiltration, score 1 indicates lymphocytic infiltration at the periphery or infiltration area <25%, score 2 indicates lymphocytic infiltration in 25% - 50% area, and score 3 indicates an area with > 50% of lymphocytic infiltration. For immunohistochemistry analysis, sections were processed and labeled using a standard immunoperoxidase technique. Briefly, antigens were unmasked by heat induced epitope retrieval after deparaffinization, and slides were treated with 1% H2O2 in phosphate buffered saline (PBS) for 15 min at room temperature to abolish endogenous peroxidase activity. The sections were incubated overnight with primary antibody against Beclin 1 and Caspase-3 (Abgent, USA), respectively. They were then incubated with peroxidase-labeled polymer that is conjugated with secondary antibody for 30 min. Each incubation step was followed by three washes for 5 min. Reaction products were developed with diaminobenzidine and counterstained with haematoxylin. Images were taken and analyzed by image processing software (Image-ProPlus 5.0), and the numbers of positive cells were counted. The rates of Beclin 1-positive or Caspase-3-positive cells were graded as the following: (-) when less than 20% of the cells were positive; (+) when 20-40% of the cells were positive; (++) when 40-75% of the cells were positive; (+++) when more than 75% of the cells were positive. The results were reviewed independently by two pathologists.
**Electron microscopy evaluation**— Mice pancreas, kidney, spleen and thymus were fixed with 4% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C for 2 h. After rinsing with PBS and post-fixation with 1% osmium tetroxide, they were dehydrated through a series of graded acetone washes. Samples were embedded in epoxy resin, sectioned, and placed onto 200-mesh copper grids. The grids were stained with uranyl acetate and lead citrate, and samples were examined using a Hitachi transmission electron microscope.

**Western blot analysis**— Mice spleens and thymuses were minced by a grinder and resuspended with PBS, then passed through a 200 μm-mesh stainless steel sieve. Mononuclear cells (MNCs) were separated by lymphoprep (Axis-shield PoC AS, Oslo, Norway), and then their lysates were prepared using an ice-cold lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 1% Triton] containing a mixture of protease inhibitors. After determination of protein concentrations using the Micro BCA Protein Assay Reagent Kit, equivalent samples (30 μg) were resolved using 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk for 1 h and then incubated with microtubules associated protein light chain 3 (MAPLC3, LC3), Beclin1, Caspase-3, and β-actin primary antibodies at 4°C overnight. After washing with Tris buffered saline/Tween-20, membranes were incubated with the corresponding peroxidase-conjugated secondary antibody, anti-goat or anti-rabbit immunoglobulin G, for 2 h, and then developed according to the enhanced chemiluminescence system (Supersignal West DURA, Pierce). LC3-II, Beclin 1 and Caspase-3 expression was quantified with the luminescent image analyzer LAS 4000 (Fuji Film, Tokyo, Japan) and normalized to β-actin.

**Statistical analysis**— SPSS software 15.0 was used for all statistical analysis. Values are expressed as mean ± SE. Treatment effects were analyzed using Student’s t test. P<0.05 was considered significant.

**Results**

Rapamycin markedly worsened the metabolic state of diabetic mice. Blood glucose, HbA1c, total cholesterol and triglyceride values gradually increased in rapamycin-treated diabetic mice, whereas these parameters remained unchanged in diabetic controls (P<0.05). Rapamycin reduced the body weight of diabetic mice, and this was not explained by changed food intake (P<0.01). However, rapamycin did not affect body weight, food and water intake in non-diabetic mice (Tables 1 and 2).

Results demonstrated that rapamycin-treated nondiabetic mice had no significant histological alterations. Distorted islets, beta cell degranulation and loss, and infiltration of lymphocytes, monocytes and neutrophils were observed in diabetic mice. The renal sections from diabetic mice showed glomerular basement membrane thickening, inflammatory cells recruitment and accumulation of mesangial matrix. The pathologic changes of pancreas and kidney from rapamycin-treated diabetic mice were markedly worsened, their insulitis score was 2.65 ± 0.27 vs. 1.53 ± 0.16 in diabetic mice (P<0.01) (Fig. 1).

Electron microscopy studies were performed on pancreatic, renal, splenic and thymic samples. The presence of marked chromatin condensation and/or blebs was considered to be a sign of apoptosis, whereas massive vacuole accumulation and absence of chromatin condensation were treated as signs of autophagy. Autophagic vacuoles were identified from the presence of organelles and/or cytoplasmic portions surrounded by close double membranes; autophagosomes were identified from the presence of single membrane vacuoles containing organelles with signs of degradation. Results demonstrated that cell death was more frequent in rapamycin-treated diabetic mice than diabetic mice. Some cells from rapamycin-treated diabetic mice displayed massive

### Table 1—Effects of rapamycin on glycosylated hemoglobin, total cholesterol and triglyceride

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol,mg/dl</td>
<td>80.6±2.3</td>
<td>79.3±2.9</td>
<td>159.2±9.1**</td>
<td>193.7±11.7†</td>
</tr>
<tr>
<td>Triglyceride,mg/dl</td>
<td>60.5±7.4</td>
<td>61.9±5.1</td>
<td>98.5±11.1**</td>
<td>128.3±9.7††</td>
</tr>
<tr>
<td>Blood HbA1c,%</td>
<td>4.9±0.4</td>
<td>5.2±0.8</td>
<td>8.1±2.2*</td>
<td>14.9±5.2††</td>
</tr>
</tbody>
</table>

Gr. I= nondiabetic; Gr. II= rapamycin-treated nondiabetic; Gr. III= diabetic; Gr. IV= rapamycin-treated diabetic
HbA1c= glycated hemoglobin A1c

P values: *<0.05; **<0.01 vs Gr. I † <0.05; †† <0.01 vs Gr. III
vacuole accumulation and chromatin condensation simultaneously, indicating both autophagy and apoptosis altered (Fig. 2). The effects of rapamycin on the expression of Beclin 1, LC3-II (marker of autophagy), and Caspase-3 (marker of apoptosis) were evaluated. The data suggested that rapamycin markedly increased the expression of these proteins in diabetic mice. In immunohistochemistry assay, both Beclin 1 and Caspase 3 positive cells increased from 30 (+) to 45% (++) when treated diabetic mice with rapamycin ($P<0.05$) (Fig. 3). Western blot analysis also showed that the level of Beclin 1 and LC3-II was up-regulated in rapamycin-treated diabetic mice ($P<0.05$) (Fig. 4).

**Table 2—Effects of rapamycin on glucose, body weight, water and food intake**

<table>
<thead>
<tr>
<th>Day</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>

[Values are mean ±SE from 10 animals in each group]

Gr. I= nondiabetic; Gr. II= rapamycin-treated nondiabetic; Gr. III= diabetic; Gr. IV= rapamycin-treated diabetic

A=glucose (m mol/L), B=body weight (g), C=water intake (g), D=food intake (g)

$P$ values: *$<0.05$; **$<0.01$ vs Gr. I; †$<0.05$; ††$<0.01$ vs Gr. III
Discussion

It is not clear yet whether the autophagic process is involved in the cell demise or is rather a survival response activated by a death-inducing signal. The relationship between autophagy and diabetes has recently attracted scientific attention. A basal level of autophagy was reported to be necessary for maintenance of structure, mass and function of the pancreatic beta cells, and also as an adaptive response under insulin resistance. However, conspicuous autophagy may contribute to the loss of beta cell mass. As a commonly used drug, whether...
rapamycin can affect progression or prognosis in T1DM through an autophagic pathway has not been reported in the literature.

Results of the present study showed that rapamycin markedly worsened the metabolic and inflammatory process of diabetic mice at a therapeutic dose.
An increase in autophagosome formation and in expression of LC3-II and Beclin-1 were observed, suggesting that the function of rapamycin was closely related to the induction of abnormal autophagy in target cells. mTOR is a serine/threonine protein kinase, with functions in regulation of protein synthesis and cellular growth. Under physiological circumstances, insulin binds to its receptor, and activates mTOR to inhibit cell autophagy\(^26\). In T1DM mice, the insulin-amino acid-mTOR signaling pathway is blocked, and the basal level of autophagy has been substantially enhanced due to the absence of insulin, thus the induction of autophagy by rapamycin is abnormally magnified to exceed the compatibility of the cells.

The differential effect of autophagy manipulation to apoptosis signaling has been addressed\(^{27-29}\). In this study, it is interesting to note that autophagic vacuolization was synchronized with obvious apoptosis (Fig. 2). This gives the evidence that both autophagy and apoptosis pathways were activated in the same cell. Since autophagy is considered to precede apoptosis, it may be speculated that the autophagy activation promoted the apoptotic process upon rapamycin treatment in the T1DM mouse model.

Tanemura et al\(^2\) reported that rapamycin treatment of islets induced autophagy \emph{in vitro}, and the present study provided with the similar effects \emph{in vivo} in T1DM model for the first time, which may be potential in clinical practice. It has been assumed that the use of rapamycin alone or in combination with insulin prophylactically could prevent the onset, arrest the development, or retard the progression of T1DM in mammal\(^6,7\), and Bussiere et al\(^4\) suggested that new immunosuppressive drugs and tolerance induction protocols should be investigated to replace rapamycin for more effective immunosuppression.

Results of this study implies that rapamycin may regulate autophagy and apoptosis in cells of diabetic mice via affecting the expression level of Caspase 3 involved in mitochondria apoptotic pathway. In the T1DM mouse model, the role of rapamycin plays seems in two ways. On one hand, it reduces the immune assault to islet by inducing autophagy and apoptosis of the invading immune cells (especially lymphocytes); on the other hand, it may directly affect beta cells to undergo controlled suicide through conspicuous autophagy. The precise mechanisms of autophagy induced by rapamycin and the role it plays in regulation on diabetes need to be studied more substantially in future.

**Acknowledgement**

This work was supported by National Science Foundation of China (No.30671784), Postdoctoral Science Foundation of China (No. 20080430178) and Natural Science Foundation of Jiangsu Province High Education Institute (No. 08KJB310009).

**References**

15. Motyl K & McCabe LR, Streptozotocin, Type I diabetes severity and bone, \emph{Biol Proced Online}, 2009; Mar 6. [Epub ahead of print]