Rapamycin Has a Deleterious Effect on MIN-6 Cells and Rat and Human Islets

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Rapamycin (sirolimus) is a macrolide fungicide with immunosuppressant properties that is used in human islet transplantation. Little is known about the effects of rapamycin on MIN-6 cells and islets. Rapamycin had a dose-dependent, time-dependent, and glucose-independent deleterious effect on MIN-6 cell viability. At day 1, using the MTT method, 0.01 nmol/l rapamycin reduced cell viability to 83 ± 6% of control ($P < 0.05$). Using the calcein AM method, at day 2, 10 nmol/l rapamycin caused a reduction in cell viability to 73 ± 5% of control ($P < 0.001$). Furthermore, 10 and 100 nmol/l rapamycin caused apoptosis in MIN-6 cells as assessed by the transferase-mediated dUTP nick-end labeling assay. Compared with control, there was a 3.1 ± 0.6-fold increase ($P < 0.01$) in apoptosis in MIN-6 cells treated with 10 nmol/l rapamycin. A supra-therapeutic rapamycin concentration of 100 nmol/l significantly impaired glucose- and carbachol-stimulated insulin secretion in rat islets and had a deleterious effect on the viability of rat and human islets, causing apoptosis of both α- and β-cells. *Diabetes* 52:2731–2739, 2003

Rapamycin (sirolimus) is a macrolide fungicide with potent antimicrobial, immunosuppressant, and antitumor properties. As an immunosuppressant, it has a mechanism of action distinct from that of cyclosporine, tacrolimus, corticosteroids, and azathioprine. In humans, it has been used successfully as an immunosuppressant in islet (1), combined kidney-pancreas (2), renal (3), and liver (4) transplantation and as rescue therapy in lung and heart transplantation (5). Rapamycin has also found a role as a tool to suppress neointimal hyperplasia of rapamycin-eluting coronary stents in humans with coronary artery disease (6) and in the porcine coronary model (7). Indeed, gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens reveals upregulation of FK506-binding protein 12 (FKBP12), the intracellular binding protein of rapamycin (8). Furthermore, CCI-779 (a water-soluble ester analog of rapamycin) has significant activity against a wide range of in vitro human cancers. It is currently undergoing clinical evaluation as an antitumor agent (9).

The antiproliferative effects can be explained by understanding the intracellular mechanism of action of rapamycin. Rapamycin binds intracellular FKBP12 to form a complex, which binds to and inhibits the serine/threonine kinase activity of the mammalian target of rapamycin (mTOR) (10). Inhibition of mTOR blocks downstream phosphorylation of several proteins. A reduction in the kinase activity of $p70^{s6k}$ results in reduced phosphorylation of the 40S ribosomal protein S6 (which is essential for mRNA translation and hence protein synthesis) (11). Also, there is increased binding of the dephosphorylated eukaryotic initiation factor, 4E binding protein-1, or phosphorylated heat- and acid-stable protein, to the mRNA cap-binding subunit of the eukaryotic initiation factor-4 complex, which inhibits its activity. This blocks the translation of mRNAs required for cyclin D1 synthesis, protein synthesis, and cell cycle progression from G_1 to S phase (12). In addition, there is increased turnover of cyclin D1 at the protein and mRNA level. This effect, in addition to the decreased translation of cyclin D1 (due to 4E binding protein-1/eukaryotic initiation factor-4 inhibition) results in a relative deficiency of cyclin D1, which is required for G_1-to-S phase transition (9).

Rapamycin causes cell death by apoptosis in BKS-2 lymphoma cell lines. Tacrolimus, which competes with rapamycin for FKBP12 binding sites, inhibits rapamycin-induced apoptosis, suggesting that, in this cell line, rapamycin binding to FKBP12 is essential for programmed cell death (13). In addition, rapamycin induces apoptosis in the Rh1 and Rh30 rhabdomyosarcoma cell lines, and it is likely that mTOR has a critical role in rapamycin-induced apoptosis (14). Rapamycin enhances apoptosis and increases sensitivity to cisplatin in the human promyelocytic leukemia cell line HL-60 and the human ovarian cancer cell line SKOV3 (15). Rapamycin can evoke apoptosis in human dendritic cells, in a time- and dose-dependent manner (16). The caspase inhibitor, ZVAD-fmk, only partially inhibits rapamycin-induced apoptosis. Monocytes, macrophages (either monocyte-derived or freshly isolated alveolar macrophages), and myeloid cell lines are resistant to the

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DMEM, Dulbecco's modified Eagle's medium; EthD-1, ethidium homodimer; FKBP12, FK506-binding protein 12; IC_{50}, half inhibitory concentration; mTOR, mammalian target of rapamycin; PI, propidium iodide; TUNEL, transferase-mediated dUTP nick-end labeling.

apoptotic effect of rapamycin (16). In addition, interleukin-7 prevents apoptosis of T-cell acute lymphoblastic leukemia cells by downregulating the cyclin-dependent kinase inhibitor $p27^{kip1}$ and upregulating bcl-2 expression. The effect on $p27^{kip1}$ is inhibited by rapamycin, suggesting that in its phosphorylated form, $p27^{kip1}$ acts as a tumor suppressor gene (17). In addition, rapamycin inhibits mitochondrial-based $p70^{s6k}$, which prevents phosphorylation of serine-136 on the proapoptotic BAD (the phosphorylated form is inactive) and blocks cell survival induced by IGF. Furthermore, IGF-1–induced phosphorylation of BAD Ser-136 is abolished in $p70^{s6k}$-deficient cells. This suggests that $p70^{s6k}$, by phosphorylating and hence inactivating BAD, is crucial to continued cell survival (18). Although rapamycin inhibition of mTOR and subsequent dephosphorylation of $p70^{s6k}$ resulted in a significant reduction in proliferation of BxPC3 and Panc-1 human pancreatic adenocarcinoma cell lines, rapamycin alone did not induce apoptosis in this cell line (19). In summary, rapamycin has been shown to induce apoptosis in some cell lines but not others.

Brittle type 1 diabetes is increasingly being treated by human islet transplantation. In the Edmonton study (1), the immunosuppressant regime included rapamycin, low-dose tacrolimus, and daclizumab (a monoclonal antibody against interleukin-2 receptor) but not corticosteroids or cyclosporine. This regime was chosen because it is thought that these drugs, including rapamycin, have little net effect on β-cell function and peripheral sensitivity to insulin, which is an essential requirement for diabetic patients receiving human islet transplants.

In the Edmonton study, blood rapamycin levels were monitored to maintain them in the range of 12–15 ng/ml for the first 3 months and in the range of 7 to 10 ng/ml (or 7.66–10.9 nmol/l) thereafter. Another study (20) describes a putative rapamycin therapeutic window in a renal transplant regime that includes cyclosporine as 5–15 ng/ml (or 5.47–16.4 nmol/l).

The Edmonton study was not designed to assess the effect of rapamycin on islet function. A previous study (21) demonstrated that rapamycin, with or without cyclosporine, is not associated with adverse impact on islet function or glucose metabolism in the canine model of pancreatic transplantation. Another study (22) demonstrated that rapamycin induces primary nonfunction of islet xenografts in a dose-dependent manner, suggesting that rapamycin may be diabetogenic. Yet another study (23) demonstrated that rapamycin reduces insulin secretion of HIT-T15 cells after 48 h of culture, but rapamycin had no effect on insulin secretion of Wistar rat islets after 24 h of culture. The effect of rapamycin on the MIN-6 cells and islets is not clear. Thus, the aim of our study was to address whether rapamycin has any effect on MIN-6 cells and rat and human islets in vitro.

RESEARCH DESIGN AND METHODS

MIN-6 cell culture. MIN-6 insulinoma cells were cultured in T175 cm flasks in 25-mmol/l glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine at 37°C under conditions of 95% air and 5% CO_2. The medium was changed every 3 or 4 days. Cells were trypsinized weekly and passages 35–50 were used exclusively.

Isolation of pancreatic islets. The Children's Hospital of Philadelphia guidelines for the use and care of laboratory animals were followed. Male Sprague-Dawley rats (Charles River Laboratories, Boston, MA) were injected with nembutal (0.05 mg/g rat). After the rats were anesthetized, the bile duct was cannulated, and the pancreas was inflated with ~20 ml of Hanks' balanced buffer. The inflated pancreas was removed and cleaned of its lymph nodes, fat, blood vessels, and bile duct. Tissue was digested with collagenase P (Roche Molecular Biochemicals) as previously described (24,25) and purified on a discontinuous Ficoll gradient. Isolated islets were washed and cultured in complete CMRL-1066 (supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) culture medium at 37°C (95% air and 5% CO_2).

TABLE 1
Characteristics of organ donors and human islet preparations

Islet preparation	Age (years)	Sex (M or F)	Purity (%)
T71	46	M	90
T72	33	F	90
T75	17	F	60
T103	58	F	40

MIN-6 cell viability determination.

MTT. The MTT assay is an indirect measure of cell viability. The assay is based on the ability of viable cells to reduce MTT (C,N-diphenyl-N′4-5-dimethyl thiazol-2-yl tetrazolium bromide) to insoluble colored formazan crystals. MIN-6 cells (1.6×10^6) from one T175 flask were plated in one 24-well plate in 25-mmol/l glucose DMEM for 3–4 days. Thereafter, the medium was removed and replaced with different glucose concentrations and different rapamycin concentrations in DMEM for either 1, 2, or 4 days. After culture with or without rapamycin, cells were washed twice with 1 ml Krebs-HEPES buffer (115 mmol/l NaCl, 24 mmol/l NaHCO3, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, and 25 mmol/l HEPES, pH 7.4, and 1% BSA) with no glucose and incubated in 1 ml Krebs-HEPES buffer (0 mmol/l glucose) with 0.5 mg/ml MTT for 60 min at 37°C. The supernatant was discarded, and cells in each well were lysed with 500 μl of 2-propanol and incubated for 60 min at room temperature. The optical density of the resultant colored 2-propanol was measured at 560 nm on a MicroKinetics plate reader.

Fluorescent live/dead cell assay. A two-color fluorescence cell viability assay was used based on the ability of calcein AM to be retained within live cells, inducing an intense uniform green fluorescence and ethidium homodimer (EthD-1) to bind the nuclei of damaged cells, thus producing a bright red fluorescence in dead cells. MIN-6 cells (1.6×10^6) from one T175 flask were plated in one 24-well plate in 15-mmol/l glucose DMEM for 3–4 days. Thereafter, the medium was removed and replaced with different rapamycin concentrations in 15-mmol/l glucose DMEM for either 2 or 4 days. After culture with or without rapamycin, cells were washed twice with 0.5 ml sterile PBS. The wash solution was then centrifuged, the supernatant removed, and the pellet resuspended in 200 μl PBS containing 2 μmol/l calcein AM and 4 μmol/l EthD-1. This solution was then returned to the appropriate well of the 24-well plate and incubated at room temperature for 45 min in darkness. The green fluorescence of the live cells was measured by the Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Gaithersburg, MD). Excitation was at 488 nm, and live cells were detected at a wavelength of 510 nm with a bandpass filter. Images of the stained cells were captured by digital fluorescence microscopy using the SimplePCI software (Compix Imaging Systems, Cranberry, PA).

Cell viability in human islets. Human islets were obtained, with the approval of the local ethics committee, from the Islet Isolation Core of the Juvenile Diabetes Foundation International—Penn Islet Transplantation Center. Consent was obtained in accordance with accepted guidelines. Donor details are described in Table 1. Purity was assessed by staining the preparation with dithizone and calculating the percentage of all tissue that stained positive for insulin. Human islets were cultured in CMRL (supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and 100 units/ml penicillin) at 37°C under conditions of 95% air and 5% CO_2 in a T75 flask. All human islets were removed from the flask, washed, and resuspended in 12 ml CMRL. A volume of islet suspension was added to each well of a 6-well plate. Either vehicle control (0.1% ethanol) or rapamycin was added to each well. After 4 days culture, 0.2 ml of supernatant was removed from each well and 0.2 ml of Krebs-HEPES buffer (0 mmol/l glucose) with 0.5 mg/ml MTT was added and incubated for 120 min at 37°C. Thereafter, all well contents were removed, spun, and the supernatant was removed. The islets were then washed with 1 ml Krebs-HEPES buffer. The supernatant was again discarded and 300 μl of 2-propanol was added and incubated for 60 min at room temperature. After further centrifugation, 200 μl of supernatant was removed for MTT analysis. The optical density of the resultant colored 2-propanol was measured at 560 nm on a MicroKinetics plate reader.

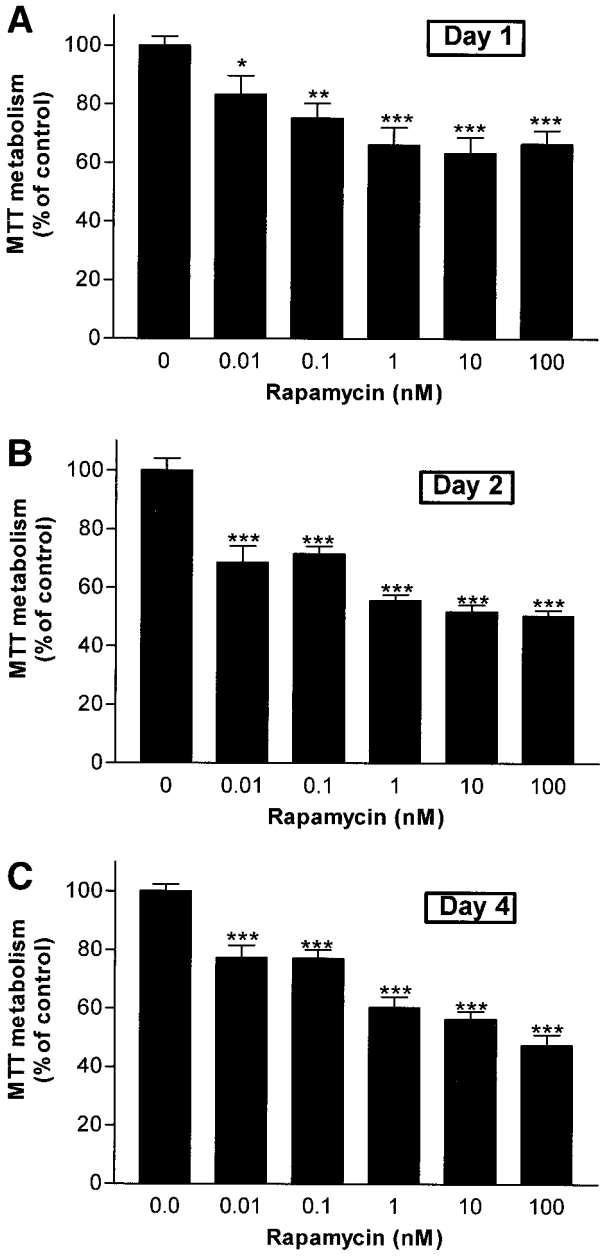

FIG. 1. Dose-dependent effect of rapamycin on MIN-6 cell viability measured by MTT metabolism. MIN-6 cells were plated in 24-well plates and cultured for 3–4 days in 25-mmol/l glucose (G25) DMEM (15% fetal bovine serum). Supernatant was removed, and cells were further cultured with different concentrations of rapamycin or vehicle control (0.1% ethanol) in G25 DMEM (15% fetal bovine serum) for 1 day (*A*), 2 days (*B*), or 4 days (*C*). Cell viability was measured by MTT metabolism as described in RESEARCH DESIGN AND METHODS. Data are mean (% of control mean) ± SE of three experiments performed in quadruplicate ($n = 12$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Rat islet insulin secretion. Rat islets were cultured in 10-cm tissue-culture dishes, containing 10 ml of supplemented CMRL-1066 and 1, 10, or 100 nmol/l rapamycin or the vehicle control (0.1% ethanol). After 4 days culture, islets were removed and washed twice in RPMI medium. Within each dish, rat islets were divided into three groups depending on their size: small, medium, or large. Five rat islets per well were counted into a 6-well plate, picked sequentially, starting from the group of largest islets to the group of smallest islets and then back to the group of largest islets and incubated in 1.5 ml of either a low-glucose RPMI solution (3 mmol/l glucose) or a high-glucose RPMI solution (20 mmol/l glucose) with 1 mmol/l carbachol at 37°C for 2 h. After incubation, the supernatant was removed and analyzed for its insulin content by radioimmunoassay (Penn Diabetes Center RIA Core).

Cell viability in rat islets. Propidium iodide (PI) is a highly polar dye, which penetrates cells with damaged membranes and stains the nuclei red. Fifty rat islets were cultured in 3 ml of supplemented CMRL-1066 and either 10 or 100 nmol/l rapamycin or the vehicle control (0.1% ethanol). After 4 days culture, the PI solution was added to each well (final concentration, 15 μg/ml) and incubated for 45 min in the dark at room temperature. Islets were then removed, centrifuged, and the supernatant was removed and discarded. The islets were resuspended in 60 μl Krebs-HEPES buffer and transferred to a 384-well plate. The intensity of PI fluorescence was measured by a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Gaithersburg, MD), with excitation at 485 nm and emission at 630 nm. Fluorescent images were captured by confocal fluorescence microscopy at a pixel size of 0.546 μm and ×20 magnification with excitation at 488 nm and emission at 610 nm (Penn Diabetes Center Biomedical Imaging Core). For each well, a minimum of 10 random size–matched islets were acquired under bright and fluorescence microscopy.

Identification of apoptosis by transferase-mediated dUTP nick-end labeling assay. An in situ Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN) was used to detect apoptotic cells. In this method, terminal deoxynucleotidyl transferase was used to catalyze the polymerization of fluorescein-labeled nucleotides to free 3-OH termini of DNA strand breaks. After 19 h culture DMEM with vehicle control (0.1% ethanol), 10 or 100 nmol/l rapamycin, MIN-6 cells were trypsinized and washed twice with cold PBS with 1% BSA. Cells were then fixed with 200 μl 2% paraformaldehyde and incubated for 60 min at room temperature. After rinsing with PBS, cells were resuspended with 250 μl of permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and incubated for 30 min at room temperature. Then, 50 μl of transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture was added to samples and positive controls (50 μl of label solution only was added to negative controls), and cells were incubated at 37°C for 1 h. Apoptotic cells were identified by fluorescein isothiocyanate staining, and analyzed by flow cytometry. A Coulter EPICS Elite Flow cytometer (Beckman-Coulter, Hialeah, FL) equipped with a 5-watt argon laser operated at 488 nm and 260 milliwatts output was used for all studies. Monomeric forms of the MIN-6 cells were electronically gated based on forward and side scatter measurements to exclude cell aggregates from evaluation. Fluorescence signals were collected with a photomultiplier tube configured with 550 nm dichroic and 525 nm bandpass filters. Ten thousand events were collected into a four-decalog single-parameter histogram for each sample. Percentage of positive cells were determined based on the evaluation of cells treated with TUNEL reagents lacking terminal deoxynucleotidyl transferase using a cursor setting that yielded <2% positive cells.

Islet-cell apoptosis identified by electron microscopy. Rat islets were cultured in complete CMRL-1066 in 6-cm dishes with vehicle or with rapamy-

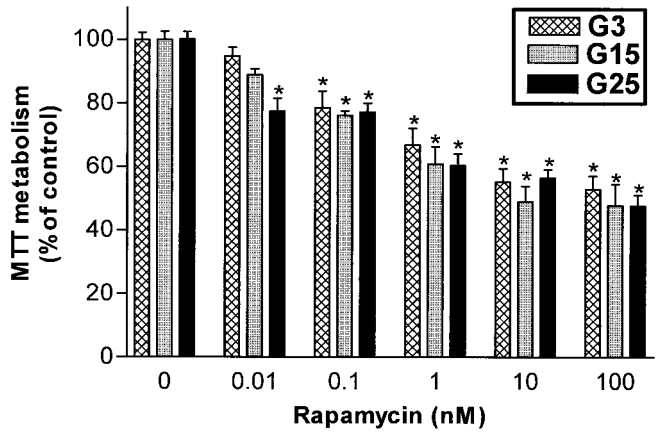

FIG. 2. Glucose-dependency of rapamycin effect on MIN-6 cell viability measured by MTT metabolism. MIN-6 cells were plated in 24-well plates and cultured for 3–4 days in 25-mmol/l glucose (G25) DMEM (15% fetal bovine serum). Supernatant was removed, and cells were further cultured with different concentrations of rapamycin or vehicle control (0.1% ethanol) each in 3-mmol/l glucose (G3, ▨), 15-mmol/l glucose (G15, □), and 25-mmol/l glucose (G25, ■) DMEM (15% fetal bovine serum) for 4 days. Cell viability was measured by MTT metabolism as described in RESEARCH DESIGN AND METHODS. Data are mean (normalized to control mean in the absence of rapamycin) ± SE of three experiments from 8 to 12 observations. In the absence of rapamycin, the absorbance for each condition was 0.236 ± 0.40 (G3), 0.354 ± 0.051 (G15), and 0.535 ± 0.018 AU (G25). *$P < 0.05$.

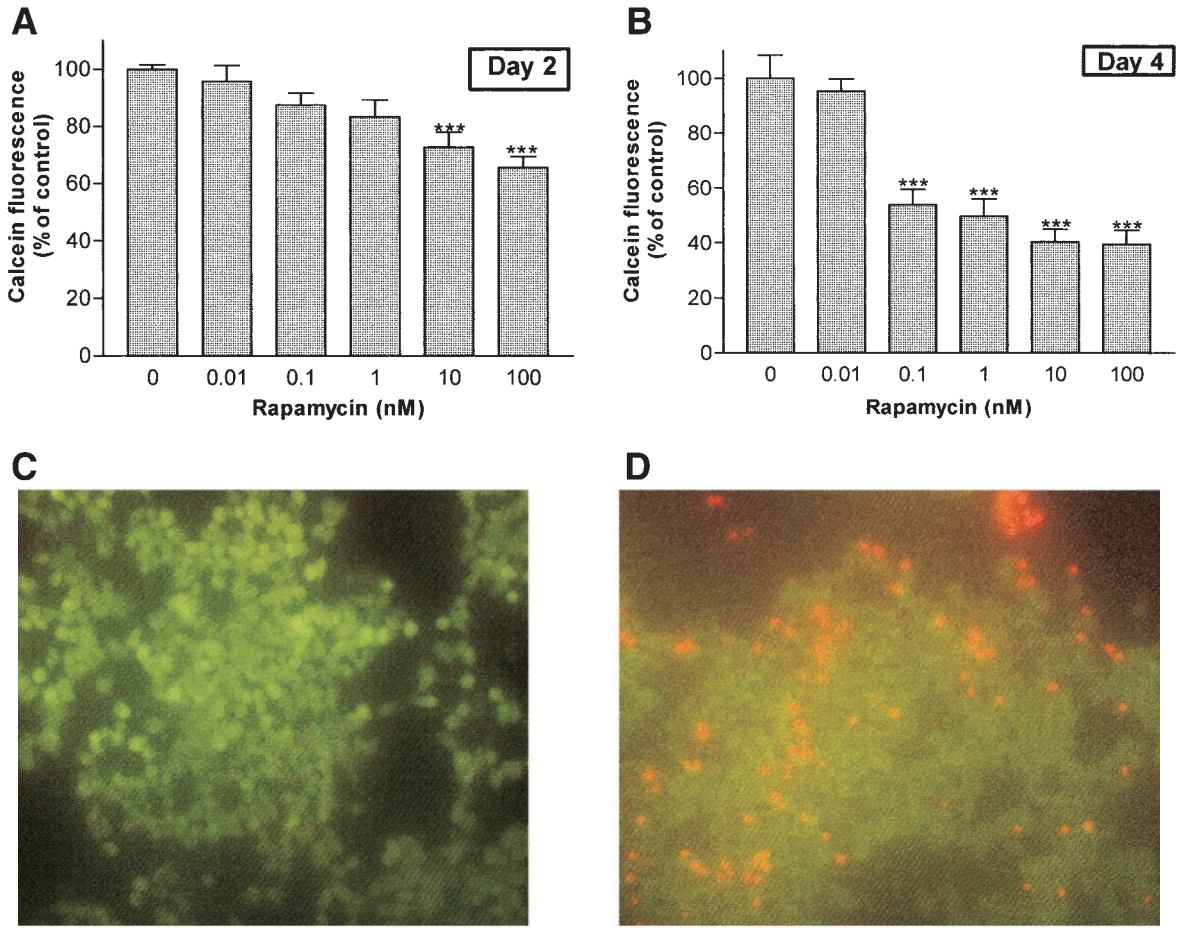

FIG. 3. Dose-dependent effect of rapamycin on MIN-6 cell viability measured by calcein fluorescence. MIN-6 cells were plated in 24-well plates and cultured for 3–4 days in 15-mmol/l glucose (G15) DMEM (15% fetal bovine serum). Supernatant was removed, and cells were further cultured with different concentrations of rapamycin or vehicle control (0.1% ethanol) for 2 days (*A*) or 4 days (*B*). Cell viability was quantitated by calcein fluorescence as described in RESEARCH DESIGN AND METHODS. Data are mean fluorescence (normalized to control mean) ± SE of three experiments performed in quadruplicate (n = 12). Fluorescent images are of MIN-6 cells cultured with 0.1% ethanol (*C*) or 100 nmol/l rapamycin (*D*) for 4 days, then stained with calcein AM and EthD-1. ***P < 0.001.

cin 100 ng/ml for 4 days. Islet preparations were then washed briefly with prewarmed serum-free medium and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer for 4 h. The islets were collected from the dish to make a pellet. After washing, osmication, and dehydration with ethanol and propylene oxide, the pellets were embedded in EM-Bed 812 and polymerized at 70°C for 48 h. Semithin sections (1 μm) were stained with Toluidine blue to screen general cell morphology. Ultrathin sections (80 nm) were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL-1010 transmission electron microscope operated at the accelerated voltage of 80 kv.

Statistical analysis and data presentation. Data are presented as mean ± SE. Statistical significance of differences between groups was analyzed by one-way ANOVA and Newman-Keuls multiple comparison tests. The molecular weight of rapamycin is 914.2, thus a rapamycin concentration of 10 ng/ml is actually 9.142 nmol/l. The rapamycin stock solutions used in this study were made up to give 0.01, 0.1, 1, 10, and 100 ng/ml. However, to ease comparisons with the units used in clinical therapeutic drug monitoring, the rapamycin concentration data in the graphs are labeled as 0.01, 0.1, 1, 10, and 100 nmol/l.

RESULTS

Dose-dependent effect and time-course of rapamycin on MIN-6 cell viability using MTT. To determine whether rapamycin has a deleterious effect on MIN-6 cells, MIN-6 cells were incubated with rapamycin concentrations of 0 (vehicle control), 0.01, 0.1, 1, 10, and 100 nmol/l in DMEM for 1, 2, or 4 days and then evaluated for cell viability using the MTT method. As early as day 1 (Fig. 1*A*), rapamycin started to reduce MIN-6 cell viability in a dose-dependent manner. At day 1, the threshold concentration causing decreased MIN-6 cell viability was 0.01

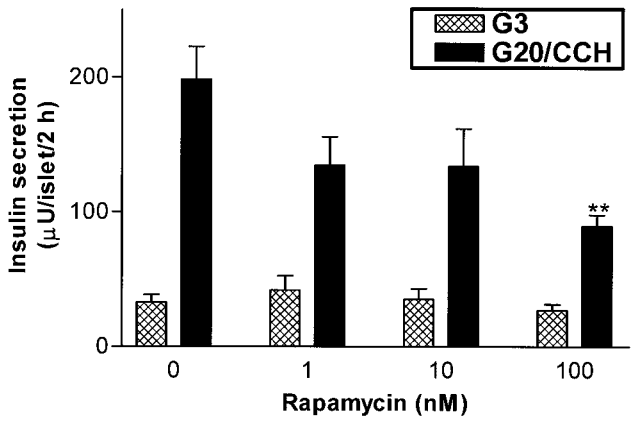

FIG. 4. Effect of rapamycin on rat islet insulin secretion after 4 days of culture. Rat islets were cultured with either 1, 10, or 100 nmol/l rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Islets were washed and then incubated for 2 h with 3 mmol/l glucose (G3) or 20 mmol/l glucose and 1 mmol/l carbachol (G20/CCH). Samples were then taken for insulin radioimmunoassay. Data are mean insulin secretion ($\mu U \cdot islet^{-1} \cdot 2\ h^{-1}$) ± SE in four experiments from 8 to 12 observations. **P < 0.01.

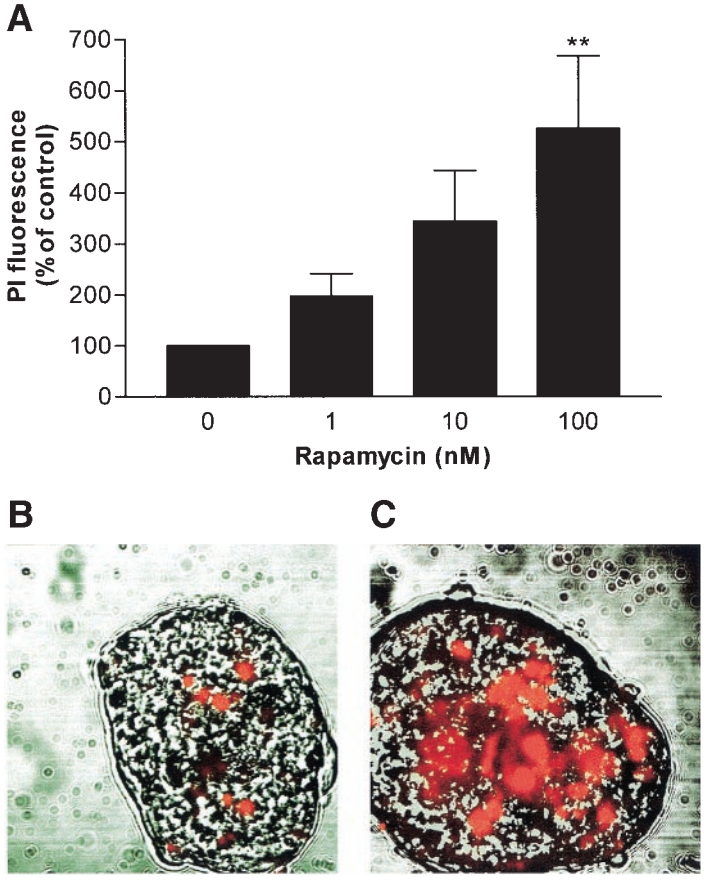

FIG. 5. Effect of rapamycin on rat islet cell viability. *A*: Rat islets were cultured with either 1, 10, or 100 nmol/l rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Islets were then stained with PI and the fluorescence intensity measured as described in RESEARCH DESIGN AND METHODS. Data are mean fluorescence (normalized to control mean) ± SE in six experiments. Rat islets were cultured with 0.1% ethanol (*B*) or 100 nmol/l rapamycin (*C*) for 4 days and then stained with PI. A bright field image was first acquired followed by a confocal image to detect PI fluorescence and overlaid. **$P < 0.01$.

nmol/l rapamycin (83 ± 6% of control, $P < 0.05$), the maximal effect was at 10 nmol/l rapamycin (63 ± 5% of control, $P < 0.001$), and half inhibitory concentration (IC_{50}) was 0.02 nmol/l rapamycin. At day 2 (Fig. 1*B*), the threshold concentration was 0.01 nmol/l rapamycin (68 ± 6% of control, $P < 0.001$), the maximal effect was at 10 nmol/l rapamycin (52 ± 2% of control, $P < 0.001$), and IC_{50} was 0.01 nmol/l rapamycin. At day 4 (Fig. 1*C*), the threshold concentration was again 0.01 nmol/l rapamycin (77 ± 4% of control, $P < 0.001$). The maximal effect was not reached at day 4 and hence the IC_{50} could not be calculated. The threshold concentration at days 1, 2, and 4 was the same (0.01 nmol/l rapamycin), although the level of significance increased from day 1 to days 2 and 4. The IC_{50} fell from 0.02 nmol/l rapamycin at day 1 to 0.01 nmol/l rapamycin at day 2. These results suggest that rapamycin has a dose-dependent deleterious effect on MIN-6 cell viability, evident at 1 day by using the MTT method.

Glucose-dependency of rapamycin effect on MIN-6 cell viability. These experiments were designed to assess whether the rapamycin effect on MIN-6 cell viability, as measured by the MTT method, was glucose-dependent after 4 days culture (Fig. 2). MIN-6 cells cultured in 3 or 15 mmol/l glucose in DMEM had a threshold concentration of 0.1 nmol/l rapamycin (78 ± 5% of control, $P < 0.05$, and 76 ± 1% of control, $P < 0.05$, respectively). MIN-6 cells cultured in 25-mmol/l glucose DMEM had a threshold concentration of 0.01 nmol/l rapamycin (77 ± 4% of control, $P < 0.05$). The maximal effect was not reached for 3 or 25 mmol/l glucose, but MIN-6 cells cultured in 15 mmol/l glucose had a maximal effect at 10 nmol/l rapamycin (49 ± 5% of control, $P < 0.05$) and an IC_{50} of 0.09 nmol/l rapamycin. At each rapamycin concentration (0.01 up to 100 nmol/l) there was no significant difference between the cell viability of MIN-6 cells cultured for 4 days in 3, 15, or 25 mmol/l glucose. These results suggest that the dose-dependent effect of rapamycin on MIN-6 cell viability is glucose independent.

Dose-dependent effect and time-course of rapamycin on MIN-6 cell viability using calcein AM. The aforementioned MTT assay is an indirect measure of cell viability—it actually measures formazan, which is a purple substance formed by the oxidation of yellow MTT by the action of mitochondrial succinate dehydrogenase. Hence, the more mitochondrial succinate dehydrogenase present, the greater the purple intensity of the sample. However, it is possible that the overall activity of succinate dehydrogenase is not only related to the number of viable cells present. It is difficult to exclude an intracellular metabolic effect of rapamycin, which may be having an inhibitory effect on succinate dehydrogenase. For this reason, we attempted to demonstrate reduced MIN-6 cell viability by a different method, a two-color fluorescence cell viability assay. This assay is based on the ability of calcein AM to be retained within live cells, inducing an intense uniform green fluorescence and EthD-1 to bind the nuclei of damaged cells. Thus the more green fluorescence present, the more live cells present in the sample. At day 2 (Fig. 3*A*), the threshold effect was at 10 nmol/l rapamycin (73 ± 5% of control, $P < 0.001$), with no maximal effect achieved. At day 4 (Fig. 3*B*), the threshold effect was at 0.1 nmol/l rapamycin (54 ± 5% of control, $P < 0.001$), with a maximal effect at 10 nmol/l rapamycin (40 ± 4% of control, $P < 0.001$) and an IC_{50} of 0.01 nmol/l rapamycin. The fluores-

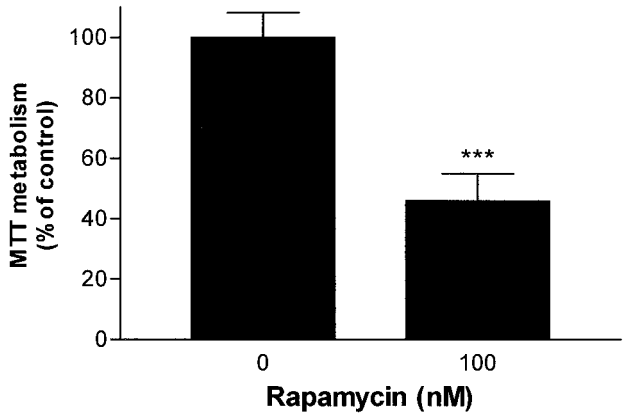

FIG. 6. Effect of rapamycin on human islet cell viability after 4 days of culture. Human islets were cultured with either rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Cell viability was measured by MTT metabolism as described in RESEARCH DESIGN AND METHODS. Data are mean (normalized to control mean) ± SE of four experiments with 11 observations. ***$P < 0.001$.

cent images of MIN-6 cells demonstrate increased bright red fluorescence in those cells treated with rapamycin (Fig. 3*D*) compared with control (Fig. 3*C*). These results confirm the previous findings, using the MTT method, that rapamycin does indeed have a dose-dependent deleterious effect on MIN-6 cell viability.

Effect of rapamycin on rat islet insulin secretion. To determine whether rapamycin has any effect on glucose-stimulated insulin secretion of rat islets, they were cultured with vehicle control (0.1% ethanol) or different concentrations of rapamycin for 4 days, and thereafter a 2-h static incubation test was performed on each group of islets. The rat islets cultured in vehicle control (0.1% ethanol) had a fourfold increase in insulin secretion in response to 20 mmol/l glucose and 1 mmol/l carbachol compared with 3 mmol/l glucose control (Fig. 4). At rapamycin concentrations of 1 and 10 nmol/l, there was a reduction in glucose-induced insulin secretion, which was not statistically significant. At a supra-therapeutic rapamycin concentration of 100 nmol/l, however, there was a 54% reduction (from 198 ± 25 to 90 ± 8 $\mu U \cdot islet^{-1} \cdot 2\ h^{-1}$) in glucose and carbachol-induced insulin secretion ($P < 0.01$). Basal insulin secretion (3 mmol/l glucose) was not affected by rapamycin at any concentration tested. These results suggest that a supra-therapeutic rapamycin concentration of 100 nmol/l significantly impairs glucose-induced insulin secretion.

Effect of rapamycin on rat and human islet cell viability. To determine whether rapamycin has any effect on human and rat islet cell viability, islets were cultured with vehicle control (0.1% ethanol) or rapamycin for 4 days, and then cell viability was measured by different methods. After 4 days culture in different rapamycin concentrations, rat islets were stained with propidium iodide to quantify rat islet cell death (Fig. 5*A*). At 1 and 10 nmol/l rapamycin, there was a small, insignificant increase in PI fluorescence, but at a supra-therapeutic rapamycin concentration of 100 nmol/l, there was a 5.3-fold increase in PI fluorescence ($P < 0.01$). The confocal images of rat islets demonstrate increased PI fluorescence from the islet cultured with rapamycin (Fig. 5*C*) compared with control (Fig. 5*B*). Human islets were cultured for 4 days with a supra-therapeutic rapamycin concentration of 100 nmol/l or control, and then human islet cell viability measured using the MTT method (Fig. 6). There was a 55% reduction in MTT metabolism compared with control islets ($P < 0.001$). These results suggest that a supra-therapeutic rapamycin concentration of 100 nmol/l has a deleterious effect on rat and human islets.

Rapamycin induces apoptosis in MIN-6 cells. To assess whether rapamycin induces apoptosis, MIN-6 cells were treated with either vehicle control (0.1% ethanol) or 10 or 100 nmol/l rapamycin for 19 h, and then the degree of apoptosis was measured by the TUNEL assay (Fig. 7). Compared with control, there was a 3.1 ± 0.6 fold increase ($P < 0.01$) in apoptosis in the MIN-6 cells treated with 10 nmol/l rapamycin and a 3.4 ± 0.4 fold increase ($P < 0.01$) in apoptosis in the MIN-6 cells treated with a supra-therapeutic rapamycin concentration of 100 nmol/l. These results suggest that 10 and 100 nmol/l (a supra-therapeutic concentration) rapamycin induces apoptosis in MIN-6 cells.

Rapamycin induces apoptosis in rat islets. Rapamycin causes cell death of rat islet cells as assessed by electron microscopy. Rapamycin treatment of rat islets resulted in numerous apoptotic β-cells (Fig. 8*B*) as well as α-cells (Fig. 8*C*). β-Cells were identified by their specific square shape granules, and α-cells were recognized by their round dark granules by electron microscopy. Vehicle-treated islet cells had a normal ultrastructure (Fig. 8*A*). In contrast, rapamycin-treated islet cells showed typical apoptotic morphologic changes, including nuclei condensation, granule dilution, clumped and condensed mitochondria, nuclear envelopes with irregular dilation, as well as some typical apoptotic bodies (Fig. 8*D*).

DISCUSSION

We have demonstrated for the first time, by several methods, that a therapeutic concentration of rapamycin causes cell death of the transformed MIN-6 cell line and that a supra-therapeutic rapamycin concentration has a deleterious effect on primary islet cells. In MIN-6 cells, this effect starts to occur as early as day 1 with a very low

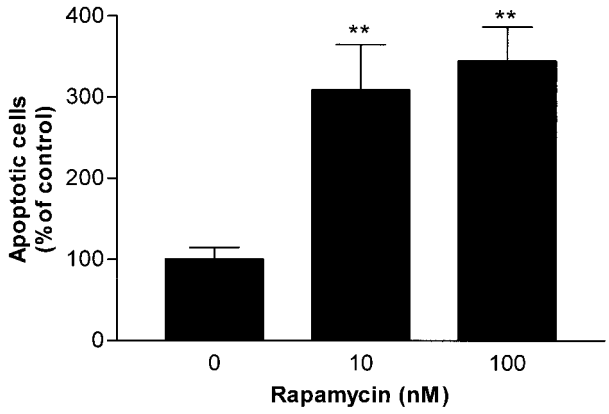

FIG. 7. Effect of rapamycin on MIN-6 cell apoptosis. MIN-6 cells were cultured with either 10 or 100 nmol/l rapamycin or vehicle control (0.1% ethanol) in 5-mmol/l glucose DMEM for 19 h, then apoptotic cells were measured by flow cytometric TUNEL assay as described in RESEARCH DESIGN AND METHODS. Data are mean (normalized to control mean) ± SE of four experiments from seven to eight observations. **$P < 0.01$.

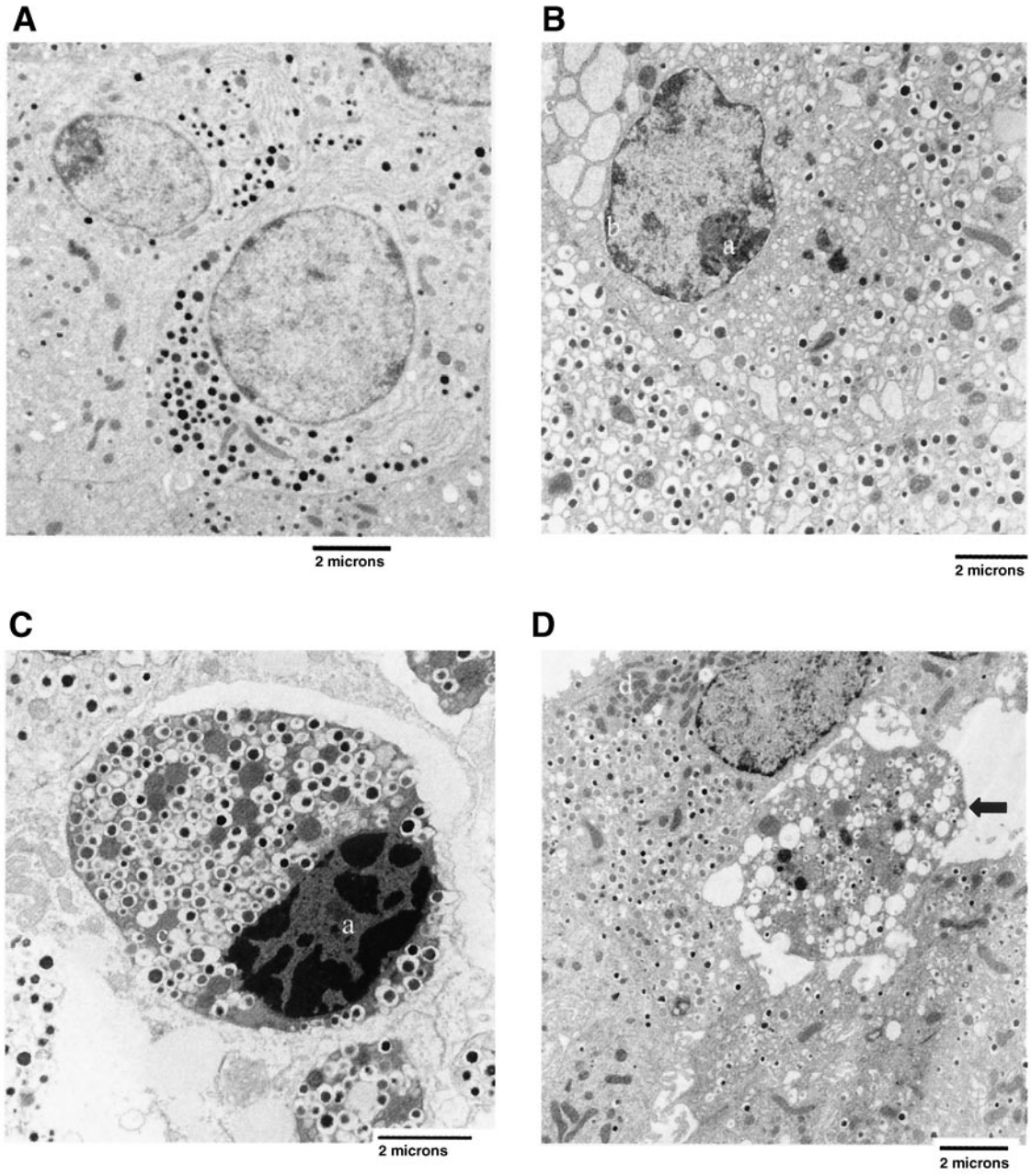

FIG. 8. Effect of rapamycin on islet-cell apoptosis. Rat islets were cultured in CMRL-1066 (10% fetal bovine serum). Islets were treated with vehicle (*A*) or with rapamycin 100 ng/ml (*B*, *C*, and *D*) for 4 days. Cell apoptosis was assessed by electron microscopy as described in RESEARCH DESIGN AND METHODS. Apoptotic cells (β-cells, *B*, and α-cells, *C*) in islets were detected by nuclear condensation (a), nuclear envelope irregular dilation (b), granule dilution (c), clumped and condensed mitochondria (d), and apoptotic bodies (solid arrows, *D*). Data are representative of two independent experiments.

threshold concentration (0.01 nmol/l), as measured by the MTT method. Why the MTT method appears to be more sensitive than the calcein method (at day 2, the threshold concentration is 10 nmol/l) is not easily explained. This may reflect the fact that calcein staining is indicative of cell death only, whereas MTT measurement reflects two cellular processes, namely cell viability and mitochondrial metabolism. As such, rapamycin may not only reduce cell viability but also have an additional inhibitory effect on mitochondrial metabolism. The effect of rapamycin does not seem to be modulated by glucose concentration in the medium.

To confirm the findings observed in the clonal insulin-producing MIN-6 cells, the effect of rapamycin was also studied in rat and human islets. The rat islet PI fluorescence experiment was designed to assess whether rapamycin causes rat islet cell damage. However, there was only a significant effect at a supra-therapeutic rapamycin concentration of 100 nmol/l. We also showed that this concentration of rapamycin reduces human islet cell viability, by the MTT method. These results confirm that rapamycin toxicity at therapeutic concentrations is limited to the MIN-6 cell line, and only at supra-therapeutic concentrations does rapamycin have a deleterious effect

on rat and human primary islet cells. The TUNEL assay data demonstrate that rapamycin-induced MIN-6 cell death occurs by apoptosis.

In addition to causing MIN-6 cell apoptosis, rapamycin also impairs insulin secretion in the static-incubation insulin-secretion test. In this study, the observed reduction in insulin secretion may be attributed in part to the reduced rat islet cell viability and in part to an intracellular signaling impairment, such as inhibition of mitochondrial glucose oxidation as possibly demonstrated indirectly by the effect of rapamycin on MTT metabolism.

The mechanisms of rapamycin-induced reduction of insulin secretion and MIN-6 cell viability are currently not completely understood. The apoptotic effects may involve the inhibition of mTOR and its downstream effectors. This may result in increased levels of phosphorylated proapoptotic factors (and tumor suppressor genes) and/or reduced levels of antiapoptotic factors. An alternative hypothesis may be a disruption of normal translation of proteins, which are essential for continued cell survival (the mTOR cell-survival pathway) (18).

The successful normalization of glucose control in brittle type 1 diabetic patients using the Edmonton Protocol has provided a potential cure for this disease. However, the long-term side effects of this protocol are still not clear, such as whether the immunosuppressants used can cause β-cell toxicity. In the landmark Edmonton Study (1), patients had trough blood rapamycin levels monitored to maintain them in the range of 12–15 ng/ml for the first 3 months after transplant and in the range of 7–10 ng/ml thereafter. It is suggested by various authors that the target therapeutic range for whole blood trough concentrations of rapamycin is 5–10 ng/ml (26) or 5–15 ng/ml (20) with concomitant cyclosporine treatment. The rapamycin concentrations required to cause in vitro MIN-6 cell death and apoptosis are similar to the range of whole blood trough rapamycin concentrations desired in patients receiving rapamycin as an immunosuppressant. The rapamycin concentration required to evoke deleterious effects on rat and human islets in our in vitro study is supratherapeutic; nonetheless, transplanted islets are also exposed to higher whole blood peak rapamycin concentrations. Furthermore, human islets transplanted into the portal vein are exposed to higher peak rapamycin concentrations, following gastrointestinal absorption of rapamycin, than the concentrations present in the circulating peripheral whole blood. Indeed, peak rapamycin levels in portal blood have been observed to be double the levels found systemically (27).

A previous study (23) demonstrated that 10 and 100 nmol/l rapamycin had no effect on rat islet insulin secretion after 24 h culture. We found that culturing rat islets with a supra-therapeutic rapamycin concentration of 100 nmol/l for 4 days did indeed reduce insulin secretion. This difference may be explained by the insufficient duration of exposure of rat islets to rapamycin in the previous study.

In summary, this is the first report to demonstrate that therapeutic concentrations of rapamycin can cause in vitro MIN-6 cell apoptosis and that supra-therapeutic concentrations of rapamycin in vitro can have a deleterious effect on rat and human islets and reduce rat islet insulin secretion. More work needs to be done on rat and human islets to establish whether therapeutic concentrations of rapamycin have any effect on primary β-cells and whether there are any consequent clinical implications.

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