Rapamycin Impairs In Vivo Proliferation of Islet Beta-Cells

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Background. Progressive graft dysfunction is commonly observed in recipients of islet allografts treated with high doses of rapamycin. This study aimed at evaluating the effect of rapamycin on pancreatic islet cell proliferation in vivo.
Methods. The murine pregnancy model was utilized, since a high rate of β-cell proliferation occurs in a well-defined time frame. Rapamycin (0.2 mg/kg/day) was given to C57BL/6 mice for 5–7 days starting on day 7.5 of pregnancy. Cell proliferation was evaluated by detection of bromodeoxyuridine incorporation by immunohistochemistry.
Results. Pregnancy led to increased β-cell proliferation and islet yield with skewing in islet size distribution as well as higher pancreatic insulin content, when compared to that of nonpregnant females. These effects of pregnancy on β-cell proliferation and mass were significantly blunted by rapamycin treatment. Minimal effect of rapamycin was observed on islet function both in vivo and in vitro. Rapamycin treatment of islets in vitro resulted in reduced p70s6k phosphorylation, which was paralleled by increased ERK1/2 phosphorylation.
Conclusions. Rapamycin treatment reduces the rate of β-cell proliferation in vivo. This phenomenon may contribute to impair β-cell renewal in transplanted patients and to the progressive dysfunction observed in islet graft recipients.

Keywords: Islets of Langerhans, Beta-cells, Proliferation, Rapamycin, mTOR, Pregnancy, Mice, Islet transplantation.

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Rapamycin is an immunosuppressive drug commonly used for the treatment of allogeneic transplant patients, including recipients of islets of Langerhans (*1*, *2*). After the success of the Edmonton Protocol of immunosuppression (i.e., high target trough levels of rapamycin and low doses of tacrolimus) (*1*, *2*), rapamycin has become part of the standard treatment in islet transplantation (*2*). Its effectiveness in preventing allorejection and autoimmunity (*3*) and the potential for promoting transplantation tolerance and regulatory T-lymphocyte survival (*4*, *5*) have all contributed to its current widespread use.

Recent reports show that patients treated with the Edmonton Protocol suffer gradual deterioration of metabolic profile requiring reintroduction of exogenous insulin (albeit at lower doses than pretransplant) (*6*, *7*). While the causes leading to progressive graft dysfunction are not yet fully understood, it is conceivable that toxicity of immunosuppressive drugs may play an important role to it (*8*).

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The effects of calcineurin inhibitors on islet function and proliferation have been recognized (*9*, *10*), while increasing data suggests that rapamycin alone (*11–13*) or in combination with tacrolimus (*14*) may impair islet cell function and survival. Furthermore, the antiangiogenic and antiproliferative properties of rapamycin (*15*, *16*) may prevent vascularization of transplanted islets, thereby reducing the mass of islets engrafting and surviving after transplantation (*15*).

Rapamycin exerts its immunosuppressive effects by inhibiting the molecular target of rapamycin (mTOR), a pathway that is involved in lymphocyte proliferation (*17*, *18*). Rapamycin exerts antiproliferative effects on multiple cell types (*17*) including pancreatic ductal cells (PDC) (*19*) and endothelial cells in vitro (*15*). Additionally, islets are currently implanted in the hepatic portal system where they are exposed to levels of immunosuppressants higher than the ones targeted systemically (*8*), which may amplify unwanted effects. It is therefore conceivable that rapamycin may impair β-cell renewal after transplantation.

Cell proliferation is most probably required for the renewal of the β-cell pool (*20*, *21*). This may occur by proliferation of existing β-cells (*22*) and/or of pancreatic ductal epithelium (*23*). Increased β-cell proliferation occurs during pregnancy (*24–27*), but also after subtotal pancreatectomy (*23*) and sudden body weight increase (*28*). In order to explore the impact of rapamycin administration on β-cell proliferation, we utilized the murine pregnancy model where β-cell proliferation occurs at a high rate, to allow for meaningful observations in a reasonably short time frame. The increased metabolic demand that takes place during pregnancy leads to the proliferation of islet β-cells (*24–27*, *29–32*). Thus, this model seemed well suited to define the potential effects of rapamycin on β-cell proliferation. Our data indicates that treatment with rapamycin leads to a significant reduction in β-cell proliferation in vivo.

MATERIALS AND METHODS

Animals

Animal procedures were performed under protocols approved by local Institutional Animal Care and Use Committee. Seven- to eight-week-old C57BL/6 (B6) mice (Jackson Laboratories; Bar Harbor, ME) were housed in virus antibody-free rooms. Mating was performed by housing two females with one male in microisolated cages. The time of vaginal plug detection after mating was considered as day 0.5 of gestation and the female was separated from the male.

Treatment Groups

Animals received 1 mg/mL bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) freshly prepared and added to the drinking water (protected from light) and rapamycin (0.2 mg/kg IP daily; Rapamune, Wyeth, Philadelphia, PA) for 5–8 days, starting at day 7.5 of gestation. Rapamycin dose was chosen based on efficacy as an immunosuppressant in mice (*4*, *33*) where it reaches serum levels comparable to those effective in humans (*19*). Control animals received no treatment. Animals were humanely sacrificed by exsanguination under general anesthesia between day 12.5 and 15.5 of pregnancy.

Immunohistochemistry of the Pancreas

At day 12.5 of pregnancy, animals were sacrificed and pancreata formalin-fixed and paraffin embedded. After deparaffinization, antigen retrieval was achieved using a decloaking chamber and citrate buffer pH 6.0 (Biocare Medical, Concord, CA). Unspecific antibody binding was reduced by applying a protein block (BioGenex, San Ramon, CA). Slides were immunostained with the following antibodies: chicken anti-insulin (1:500; Abcam, Cambridge, MA), guinea pig anti-insulin (1:100; Dako, Carpinteria, CA), rabbit anti-glucagon (1:100; BioGenex), mouse anticytokeratin (1:300; Accurate), and rat anti-BrdU (1:50; Accurate, Westbury, NY). The secondary goat antibodies (1:200) were antichicken immunoglobulin (Ig) G 568, antirabbit IgG 350, antirat IgG 488, and antiguinea pig IgG 647 and antimouse IgG 568 (Molecular Probes, Eugene, OR). Islet cells were counted and the proportion of BrdU+β- and non-β-cells in islets was determined. An average of 393±55.8 islet cells were counted for each animal (n=5 per group). Immunostained sections were assessed at the Imaging Core at the Diabetes Research Institute using a fluorescence confocal laser scanning microscope (Zeiss LSM-510, Thornwood, NY).

Islet Isolation and Dissociation

Islets were isolated from B6 donors by enzymatic (Collagenase type V; Sigma-Aldrich, St. Louis, MO) digestion of harvested pancreata, followed by purification on Euroficoll gradients (Mediatech, Herndon, VA), as previously described (*33*). Islet yield was assessed by scoring size and expressing the recovery in total number of islets (IN) as well as islet equivalents (IEQ). Culture was done in CMRL-1066 medium (Gibco, Long Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L HEPES buffer (Mediatech) (*33*).

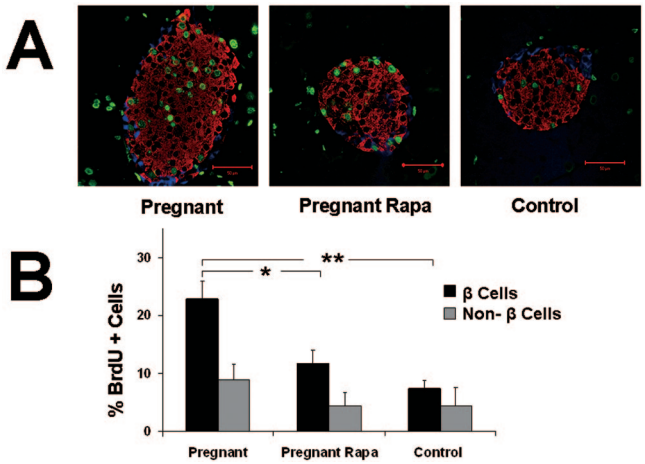


FIGURE 1. Assessment of proliferating β-cells in the pancreas of pregnant mice. (A) Proliferation of β-cells (anti-insulin antibody, red fluorescence) was assessed by immunofluorescence using anti-BrdU (green fluorescence) in the pancreas of pregnant mice (gestation day 12.5) receiving no treatment (left panel) or rapamycin (Rapa) starting on day 7.5 (central panel) as well as of nonpregnant, age-matched control female mice (right panel; 40×). Nuclei were stained with DAPI (blue fluorescence). (B) Quantification of islet cell proliferation (%) within total islet cells is shown (black bars=β-cell; gray bars=other islet cells). Data is expressed as mean±SEM of five individual animals per group (ANOVA Tukey: *P=0.017; **P<0.002).

Laser Scanning Cytometric Analysis

Islets were dissociated into single cells with Accutase (Innovative Cell Technologies, San Diego, CA) and the resulting cell suspension was fixed on glass slides using 2.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), as previously described (*34*). After antigen retrieval, slides were immunostained with rabbit anti-C-peptide (GeneTex, San Antonio, TX) and rat anti-BrdU antibodies, as well as with 4′,6-diamidino-2-phenylindoledihydrochloride (DAPI) and 7-aminoactinomycin-D (7-AAD, both from Molecular Probes). The secondary antibodies were goat anti-rabbit IgG 647 and goat anti-rat IgG 488. Data acquisition and analysis were performed using a Laser Scanning Cytometer (LSC) or the iCys Research Imaging Cytometer (CompuCyte, Cambridge, MA) (*34*) to determine the percentage of C-peptide- and BrdU-positive cells using automated fluorescence-based quantitative measurements (Olympus BX50; Melville, NY). Slides were scanned at 40×, and the nuclei were contoured by DAPI or 7-AAD staining using argon laser and blue or red detector. BrdU and C-peptide-positive events were recorded using the argon or HeNe laser and green or far-red detector. Single cells were identified and gated according to the 7-AAD or DAPI staining area. Fluorescence intensity of each computed event was recorded on a histogram. Cells from every subpopulation were visualized directly in the LSC or iCys by relocalization to confirm regular morphology (Fig. 2). The percentage of proliferating β-cells was calculated as BrdU+ signal in the C-peptide+ population. A minimum of 2000 cells were acquired and analyzed for each sample.

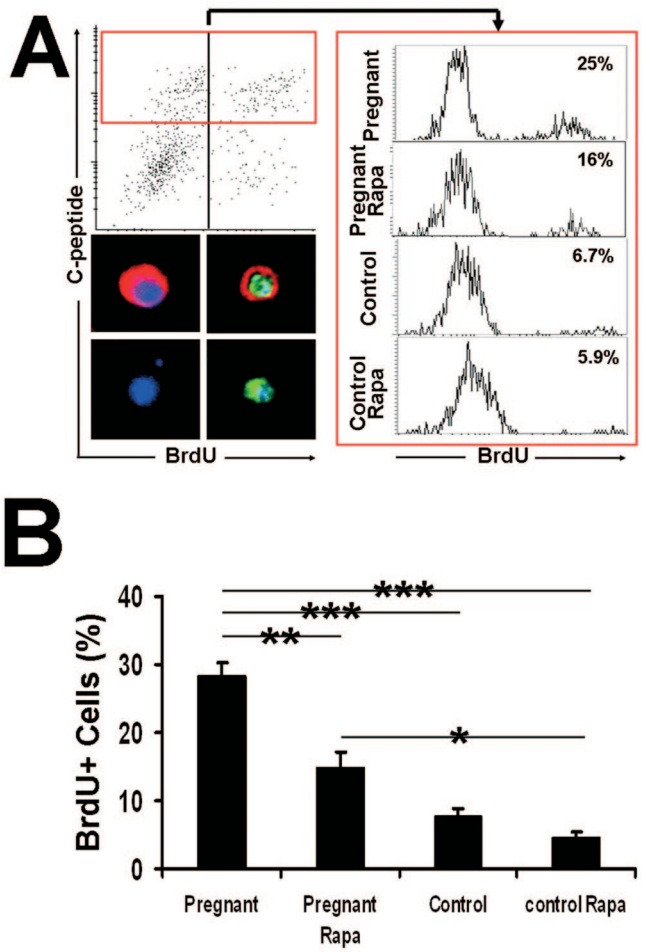


FIGURE 2. Automated quantification of β-cell proliferation in isolated islets. Islets were isolated from control and pregnant females treated or not with rapamycin (Rapa). Single islet cell suspensions were obtained from each experimental group and immunostained using specific antibodies for C-peptide (β-cells, red) and BrdU (green, nuclei); and counterstained with DAPI (blue). (A) Data was acquired using the iCys Research Imaging Cytometer after gating on C-peptide-positive cells. The percent of BrdU-positive cells in the C-peptide-positive subset in each group is shown in the four histograms of (A). Percentages for each group were calculated and are expressed numerically in this representative experiment. (B) Quantification of proliferating β-cells in the indicated experimental groups. Data is expressed as mean±SEM of at least three independent isolations per experimental group (ANOVA Tukey: *P=0.016; **P<0.003; ***P<0.0003).

Insulin Extraction

Mice were sacrificed at day 12.5 after overnight fasting and harvested pancreata were frozen at −80°C. Pancreata were homogenized in 100% ethanol and insulin extracted by two cycles of incubation with 80% phosphoric acid (Mallinckrodt, Paris, KY) at pH of 1.8 to 2.0. Supernatants were collected and kept at −80°C until assay. Insulin content was measured by enzyme-linked immunosorbent assay (ELISA; Mercodia, Winston Salem, NC). The tissue pellet was washed in phosphate-buffered saline and then kept in 0.1 M NaOH. Total proteins were measured using the BCA protein assay kit (Pierce, Rockford, IL).

Glucose Tolerance Test

After overnight fasting, mice received an intraperitoneal (IP) glucose bolus (2g/kg bw). Glycemic values were monitored in conscious animals on whole blood (obtained by tail vein puncture) using portable glucometers (OneTouch Ultra 2, LifeScan, Milpitas, CA) at selected time points for 90 min to assess glucose clearance in control and rapamycin-treated animals. Glucose disposal was measured by comparing the area under the curve (AUC) of control and experimental animals during glucose tolerance test.

Perifusion

Three independent experiments were performed where islets from pregnant mice (either untreated or rapamycin-treated) were isolated on the same day and maintained under the same culture conditions in order to minimize interexperimental variability. Glucose-stimulated insulin release studies were performed using a custom-built apparatus (Biorep Technologies, Miami, FL). Islet aliquots (100 IEQ per condition) were loaded into Perspex microcolumns containing acrylamide-based microbead slurry (Bio-Gel P-4, Bio-Rad). Krebs' buffer with selected glucose or KCl concentrations was circulated through the columns at a rate of 100 μl/min. Serial 100 μl fractions of perifusate were collected every minute. Insulin was measured by ELISA (Mercodia). Ramping perifusion was performed by intermittently exposing each column to basal (3 mM) and increasing glucose concentrations (6, 9, and 11 mM).

Evaluation of Kinase Phosphorylation

Islets were isolated from control, nonpregnant B6 mice and cultured overnight. After counting, islets were transferred to CMRL-1066 supplemented with 10% horse serum (ATCC, Manassas, VA). Rapamycin (30 ng/mL) or vehicle was added to the culture media for 50 min. At the end of the incubation, islets were washed in phosphate buffered saline, resuspended in cell lysis buffer (BioRad, Hercules, CA) and frozen at −80°C until protein extraction. Total protein concentrations were determined on islet cell lysates by BioRad DC protein assay. Assessment of the phosphorylation of protein kinases was performed by fluorescence-based quantitative measurement on a BioPlex platform (BioRad). Data was expressed as ratio of phosphoprotein to total protein and/or ratio to control islets (*35*).

Statistics

Data was analyzed using SigmaPlot 9.0 (SystatSoftware, San Jose, CA), Microsoft Excel and Statistica 6.0 (StatSoft Inc.; Tulsa, OK) and presented as mean±standard error of the mean (SEM). Comparisons between two samples were done using two-tailed paired Student's t test. Analysis of variance (ANOVA) was used for the comparison of multiple conditions using Tukey post-hoc test. Statistical significance was considered for P<0.05.

RESULTS

Rapamycin Reduces Pregnancy-Driven β-Cell Proliferation in Mice

Islet cell proliferation during pregnancy was assessed by the means of BrdU incorporation in pancreatic sections after

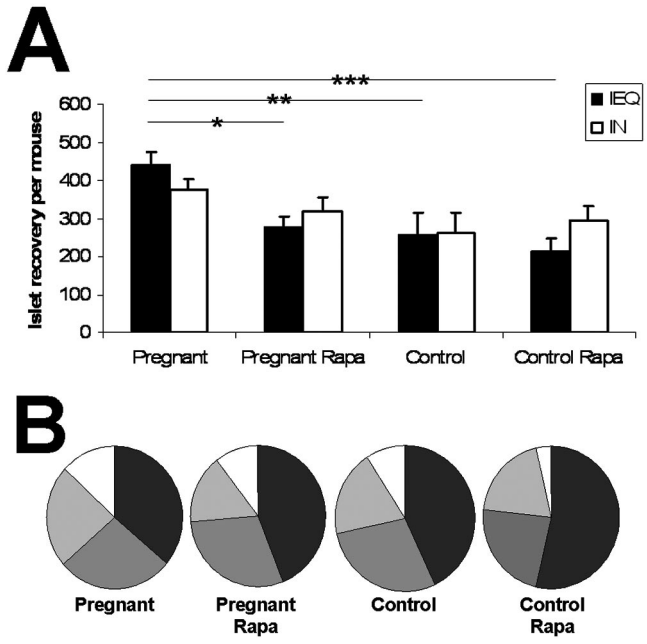
five-to-seven days of treatment (starting at day 7.5 of gestation). Proliferation of selected endocrine cell subsets was enumerated by scoring BrdU+ cells within each endocrine subset (Fig. 1A). Analysis of pancreata of pregnant mice showed that 22.8±3.1% of β-cells underwent at least one cycle of proliferation during the 5 days of BrdU administration, as demonstrated by nuclear BrdU localization in insulin-positive cells (Fig. 1B). The proportion of proliferating β-cells in rapamycin-treated pregnant animals was significantly reduced to 11.7±2.2% (P=0.017), while was 7.4±1.3% in age-matched, nonpregnant females (Fig. 1B). The proportion of BrdU+ nuclei in non-β cell subsets within islets did not differ significantly among experimental groups (Fig. 1B). Furthermore, BrdU incorporation in pancreatic ductal cells was 11.9±1.82% in control nonpregnant animals (n=6). A similar proportion of PDC proliferation was observed in pregnant mice treated or not with rapamycin (10.2±2.16 and 12.7±1.15%, respectively; n=5 per group; P=NS).

We further extended our analysis to evaluate the proliferation of islet β-cells using LSC on dissociated islet cells (Fig. 2A). This approach allows for the automated acquisition of a large number of events expressing selected fluorescent markers thereby increasing the power of analysis and reducing potential operator-dependent errors (*34*). The analysis by LSC of the proportion of BrdU+ β-cells (C-peptide positive) in dissociated islets confirmed the data obtained in the pancreatic sections and showed a significant reduction of proliferating β-cells in the rapamycin-treated pregnant animals, when compared to untreated pregnant mice (14.8±2.3% and 28.4±1.7%, respectively, P=0.002), while control females receiving or not rapamycin presented lower rates of proliferating cells (4.6±0.7%, and 7.6±1.2%, respectively; Fig. 2B). Statistically significant differences in intra-islet β-cell proliferation were observed between pregnant animals when compared to controls treated or not with rapamycin (P<0.0003), as well as between the two groups receiving rapamycin (pregnant vs. nonpregnant; P=0.016).

Effects of Rapamycin on Islet Mass

We then asked whether the observed differences in β-cell proliferation would result in significant differences in β-cell mass in experimental and control groups. Islet recovery on a per mouse basis was not different between groups when considering the IN obtained. In contrast, islet yield (IEQ/mouse) from pregnant animals treated with rapamycin was lower than untreated pregnant donors (277±26.9 vs. 441±31.8 IEQ/mouse, respectively; P=0.050; Fig. 3A). In addition, islet yield from pregnant mice was consistently higher than that of age-matched control mice (256±59.7 IEQ/mouse, P=0.008) and of rapamycin-treated mice (215±34.2 IEQ/mouse, P=0.039; Fig. 3A). The discrepancy between IN and IEQ indicates that the average islet size of pregnant mice was higher than that of other groups. This was further confirmed by comparing islet size distribution between experimental groups, which showed that pregnant mice yielded a higher proportion of large size islets, when compared to all other groups (Fig. 3B).

Assessment of total insulin extracted from the pancreas is considered a surrogate marker of islet β-cell content. Insulin content from total pancreas extracts of control mice was 5,573.9±481.8 ng (n=4) and was significantly higher in preg-



	Islet isolations	50-100µm	100-150µm	150-200µm	>200µm
Pregnant	14	36.0 ± 2.3	27.5 ± 2.8	23.6 ± 2.0	12.9 ± 1.2
Pregnant Rapa	8	43.8 ± 3.7	29.9 ± 4.1	15.7 ± 2.1	10.6 ± 1.9
Control	7	43.2 ± 3.2	28.5 ± 4.5	19.2 ± 3.8	9.1 ± 2.0
Control Rapa	4	53.5 ± 5.9	23.2 ± 3.9	19.5 ± 6.2	3.7 ± 1.5

FIGURE 3. Recovery and size distribution of isolated islets. After pancreas digestion and purification, islets obtained from pregnant or control animals treated or not with rapamycin were counted and scored by size. Islet recovery is expressed as IEQ and IN per donor mouse (A). Statistically significant differences between groups are indicated (ANOVA Tukey: *P=0.050; **P=0.008; ***P=0.039). (B) Islet size distribution in percentage is summarized. Data is representative of 7–12 individual samples from 4–11 individual islet isolations per group.

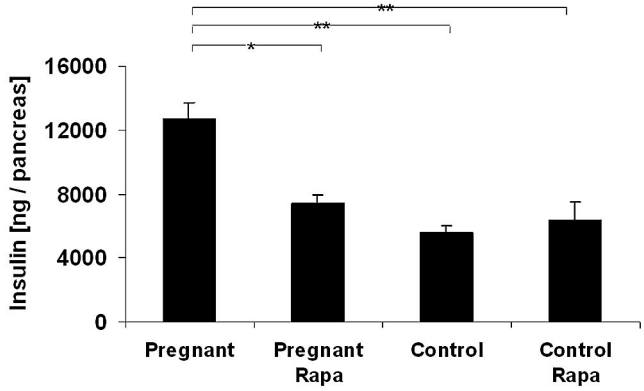


FIGURE 4. Insulin content in pancreatic tissue. Pancreata obtained from pregnant (day 12.5) and control donors treated or not with rapamycin (Rapa) were collected after overnight fasting. Insulin was extracted from whole pancreas using phosphoric acid and assessed by enzyme-linked immunosorbent assay. Data is expressed as nanograms of insulin per pancreas and presented as mean±SEM from 4–6 pancreata per experimental group (ANOVA Tukey: *P<0.002; **P<0.0009).

nant mice (12,674.2±1027.0 ng; n=6; $P<0.0008$; Fig. 4). Rapamycin treatment significantly reduced (by approximately 40%) total insulin content in the pancreas of pregnant animals (7,407.3±528.8 ng; n=6; $P=0.0017$), approaching the levels observed in nonpregnant controls (Fig. 4). Insulin content in nonpregnant animals receiving rapamycin was 6,367.1±1,159.3 ng (n=5; $P=0.008$ vs. untreated pregnant mice).

Rapamycin Does Not Grossly Affect In Vivo and In Vitro Islet Function

Assessment of fasting glycemia levels showed reduced values in untreated pregnant mice (79.7±4.1 mg/dL, n=14), when compared to nonpregnant controls (106.7±3.0 mg/dL, n=11; $P<0.02$) or rapamycin-treated nonpregnant controls (127.0±3.0 mg/dL, n=3; $P=0.005$), while was not significantly different from rapamycin-treated pregnant mice (90.4±2.8 mg/dL, n=17).

The effects of rapamycin treatment on the islet metabolic response to in vivo stimulation were assessed during a glucose tolerance test (IPGTT). Glucose clearance of control and rapamycin-treated animals in the pregnant and nonpregnant groups was comparable during the metabolic test (Fig. 5A), and no significant differences in the glycemia area under the curve (AUC) were observed between groups (Fig. 5B).

The effects of rapamycin on islet potency and glucose sensitivity were assessed on islets isolated from either rapamycin-treated or untreated pregnant donors. For each independent experiment (n=3), islets from both experimental groups were isolated on the same day and kept under the same conditions. After overnight culture, islet aliquots were exposed intermittently to 3 mM and to increasing glucose concentrations (6, 9, and 11 mM; Fig. 5C). Both islets from treated and untreated pregnant mice showed good response to glucose, with only a small reduction in insulin output observed in islets from rapamycin-treated mice (Fig. 5C). Perifusion profiles did not differ in the two groups (data not shown).

Rapamycin Treatment of Islets Results in Reduced Phosphorylation of p70s6k and Increased Phosphorylation of ERK

In order to assess the direct effects of rapamycin on islet cells, we studied the phosphorylation of p70s6k, the downstream effector of mTOR, as well as other signal transduction pathways involved in cellular survival and proliferation. Treatment of isolated islets with rapamycin in vitro led to the reduction of p70s6k phosphorylation ($P=0.02$; Fig. 6), demonstrating that rapamycin indeed exerts a direct effect on islets. In rapamycin-treated islets, phosphorylation of the extracellular-signal regulated kinase 1/2 (ERK) was increased when compared to control, untreated islets ($P=0.01$; Fig. 6). No differences were observed in the phosphorylation of AKT (Fig. 6) as well as of other kinases (i.e. p38 and IRS1), when comparing control and rapamycin-treated islets (not shown).

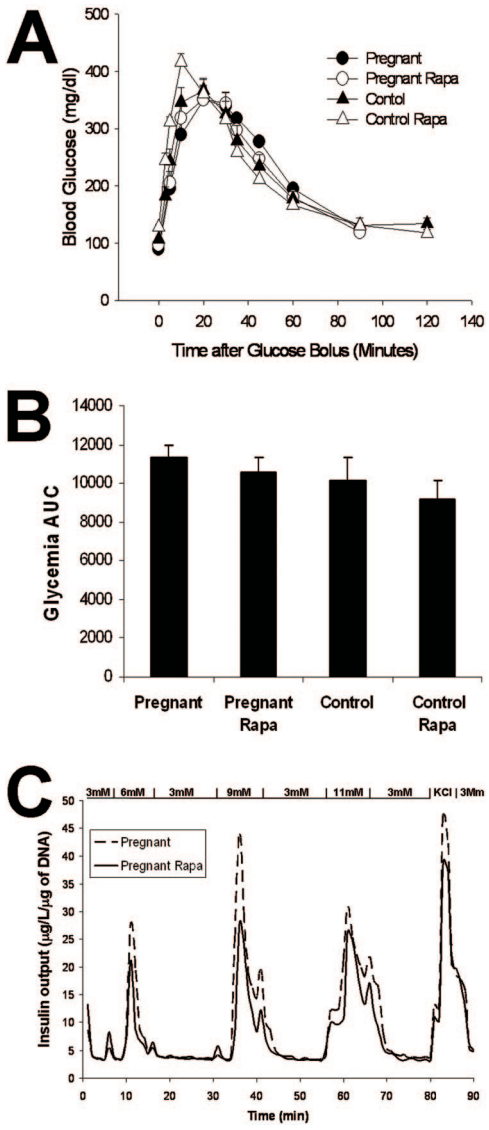


FIGURE 5. Metabolic function. (A and B) In vivo intraperitoneal glucose tolerance test. A glucose bolus (2 g/kg) was administered after overnight fasting to pregnant and control animals treated or not with rapamycin (Rapa). Glucose clearance was monitored as a function of time over a 2-hour period. Glycemic profiles (A) and area under the curve (AUC; B) for glucose from 4–6 animals per group are shown as means±SEM. (B) Dynamic glucose stimulated insulin release in vitro. Islets isolated from pregnant mice treated (solid line) or not (broken line) with rapamycin (Rapa) were stimulated in vitro (100 IEQ aliquots) with the indicated increasing glucose concentrations (indicated on top). Maximal stimulation was obtained by KCl. Insulin output was normalized to DNA content. A representative profile of three independent experiments is shown.

DISCUSSION

The main goal of the present study was to evaluate the antiproliferative effects of rapamycin on β-cells in vivo. The underlying hypothesis was that preventing physiological turnover of islet β-cells in transplanted patients by the administration of immunosuppressive drugs that block their proliferation may contribute to graft dysfunction. The occurrence of low rate β-cell proliferation under physiological conditions has been described for both rodents (*22*) and humans (*21*, *36*); it is therefore conceivable that it may also take place in the context of islet grafts although there is no data yet available on whether proliferation in such an ectopic site is

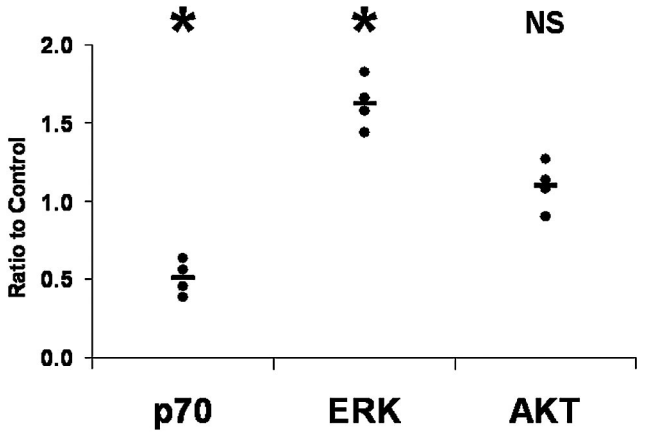


FIGURE 6. Kinase phosphorylation. Isolated islets were stimulated or not in vitro with rapamycin for 50 min. Phosphorylation of p70s6k, ERK, and AKT was assessed by BioPlex on protein extracts. Data is expressed as ratio to phosphorylation on control untreated islets of four independent islet preparations. Horizontal lines indicate the mean of the group (paired *t* test: $*P<0.03$ vs. control islets).

qualitatively and quantitatively identical to that occurring in the native pancreas. While indirect evidence of an increased islet mass occurring after transplantation in the absence of drug toxicity has been suggested by our observation in primates (*37*), direct testing of this hypothesis in patients is unfeasible due to the lack of reliable noninvasive tests to assess and monitor islet mass (i.e., metabolic and imaging), and therefore we sought to obtain valuable albeit indirect information by establishing an experimental model in small animals. Pregnancy leads to enhanced β-cell proliferation at well-defined times and appeared therefore as a suitable model to address, in a reasonable time frame, some of the questions that we wanted to pose. Even if the proliferation of β-cells occurs at a lower rate in the transplant setting, graft dysfunction is observed over a period of years, where the contribution of an even very low rate of spontaneous proliferation to β-cell mass would be revealed.

Proliferation during pregnancy has been associated with the effect of lactogenic hormones on the β-cells via prolactin (PRL) receptors (PRL-R) (*26*, *27*, *31*, *38*, *39*). Pregnancy hormones such as placental lactogen (PL), PRL, and growth hormone (GH) can induce proliferation of β-cells (*40*, *41*) via the Jak-2 /STAT-5 pathway (*31*, *38*). Additional signal transduction pathways may participate to β-cell proliferation. In particular, phosphorylation of IRS-1 and 2, Sarc homology collagen (SHC), ERK1/2, AKT, and p70s6k are increased in rodent islets during pregnancy (*30*). Furthermore, treatment of pregnant rodent islets with PRL-R antisense oligonucleotides results in the reduction of p70s6k phosphorylation (*30*).

We directly investigated the effect of rapamycin treatment on in vivo islet cell proliferation. We administered BrdU over a 5-day period in order to acquire cumulative data on proliferation events occurring during the treatment period. Our data indicates that in control, nonpregnant animals, ~7.5% of pancreatic β-cells incorporated BrdU. This is in agreement with previous reports in which exposure to BrdU for 2–4 hr showed β-cell proliferation rates of 0.2–0.3% in rodents (*42*, *43*).

Also, in keeping with previous reports, high islet β-cell proliferation (>20%) was observed during pregnancy in mice (*24–27*, *32*). A short-course treatment with rapamycin resulted in a highly significant reduction of β-cell proliferation measured as lower numbers of BrdU-labeled nuclei in pancreatic sections by immunohistochemistry that was confirmed by more sophisticated and quantitative LSC techniques on dissociated islet cells.

Pregnancy also resulted in increased pancreatic insulin content that was paralleled by higher islet recovery and larger size distribution after isolation, when compared to control animals. While the overall islet yield did not significantly differ between experimental groups when IN was calculated, the IEQ counts (which take in account both islet size and number) showed a significantly higher recovery from untreated pregnant donor pancreata, when compared to all other groups. This observation points to the presence of larger islets in the preparations that was indeed confirmed by the size distribution obtained in the counting samples.

Rapamycin treatment in pregnant mice resulted in a significant decrease in pancreatic insulin content and reduction in isolated islet yield and size, when compared to that of untreated pregnant mice. In contrast, rapamycin treatment did not significantly affect β-cell proliferation, pancreatic insulin content, or islet yield in control nonpregnant females. This can be attributed to the relatively low rate of proliferation of islet cells in basal conditions and to the short-course of rapamycin treatment in our study. Thus, the model of pregnancy offers a unique tool for the study of high β-cell proliferation under physiological conditions in a well-defined time frame.

Rapamycin acts by forming a complex with FK Binding Protein-12 (FKBP12) that binds to mTOR, thereby inhibiting its activity (*17*, *18*, *44*). The mTOR-p70s6k complex has been implicated in the control of cell proliferation, growth, and metabolism and is activated by environmental stimuli such as nutrients (i.e., amino acids and glucose), growth factors, and cellular energy status in multiple cell types (*17*, *44–46*), including islet cells (*45*, *47*). Activation of mTOR results in phosphorylation activation of the ribosomal-S6 kinase (p70s6k) leading to protein synthesis and β-cell hypertrophy (*44*, *45*, *48*, *49*), while deficiency of S6K1 is associated with hypoinsulinemia and glucose intolerance (*49*).

The increased β-cell proliferation and mass in pregnant pancreata (measured as insulin content) was associated with significantly lower fasting glycemic values than in control animals. Conversely, glucose clearance during IPGTT was comparable between the experimental groups (pregnant and controls treated or not with rapamycin). Increased metabolic demand and insulin resistance in pregnant mice are considered important triggers to the wave of β-cell proliferation (*24–27*). It is conceivable that an increased β-cell mass during pregnancy may compensate for this demand by maintaining normal glycemic control. Conversely, rapamycin can improve insulin sensitivity in peripheral tissues (*46*), an effect that may have contributed to the observed comparable glucose clearance between experimental groups in our study. Additionally, rapamycin treatment may result in improved metabolic responses in vivo and in vitro (*50*).

While β-cell proliferation dramatically decreased after rapamycin treatment, little effect was observed on in vitro function and metabolic response to glucose. In contrast with our observation, rapamycin has been reported to affect islet function both in vitro (*11*) and in vivo (*19*), as well as inducing insulin resistance (*14*). The discrepancies between previous reports and our results may be due to the substantial differences in the experimental models utilized. In particular, our in vivo treatment was relatively short (5–7 days) and it is possible that an extended rapamycin treatment would result in a measurable functional impairment in islet cells and of metabolic pathways that contribute to glucose homeostasis.

Prevention of islet β-cell proliferation by rapamycin could be explained by a direct effect on β-cells or by an indirect effect mediated via prevention of pregnancy progression or alteration of embryogenesis. This in turn may lead to reduction in pregnancy-driven β-cell proliferation. There have been reports that the administration of rapamycin in pregnancy leads to abnormal embryo development and decreased size of fetuses through a putative inhibition of the mTOR signal transduction cascade (*51*). Notably, this was observed with much higher rapamycin doses (>20-fold) administered starting earlier in pregnancy than what was used in our protocol (*51*). Our experimental design included initiation of rapamycin treatment on day 7 of pregnancy (when implantation has already occurred) and recovery of pancreatic glands after 1 week of treatment. Although in some cases smaller pups were observed in rapamycin-treated animals, no statistical differences were detected in the number of pups on a per litter basis, the size of the fetuses, and the uterus weight normalized by the number of fetuses in pregnant females treated or not with rapamycin (not shown). We therefore would like to propose that direct effects of rapamycin on islet β-cell proliferation are likely to contribute to our findings. To this regard, the observation of the reduced phosphorylation of p70s6k in islets treated in vitro with rapamycin concentrations comparable to those reached in vivo in animals and patients demonstrates a direct effect of rapamycin on islet proliferation. Rapamycin treatment of islets in vitro also resulted in a significant increase in phosphorylation of ERK1/2, while no differences in AKT phosphorylation were observed, when compared to control islets. The ERK1/2 is a subfamily of the mitogen-activated protein kinases (MAPK) involved in the intracellular signaling associated with cell survival that can also lead to phosphorylation of p70s6k (*46*). Blockade of mTOR in umbilical vascular endothelial cells results in an increased ERK1/2 phosphorylation in response to stimulation with amino acids and insulin, which suggests a putative inhibitory action of mTOR on this signal transduction pathway (*46*). Our finding of increased ERK1/2 phosphorylation in islet cells exposed to rapamycin suggests that a similar mechanism may be operational in our setting. These data point to the likely involvement of the p70s6k pathway in β-cell proliferation.

The observation that β-cell turnover occurs in physiological conditions (*20–22*) clearly suggests that the use of drugs that prevent β-cell proliferation will result, in time, in the reduction of the β-cell mass that survives. Since transplanted patients receive a mass of islets that often is likely of suboptimal size, it appears conceivable that β-cell mass attrition due to reduced proliferative capacity in the presence of rapamycin therapy will manifest itself during the course of the follow up, as it is commonly observed in the ongoing clinical trials of islet transplantation based on the Edmonton protocol of immunosuppression (*2*, *6*). Even postulating that a low level of proliferation occurs in the implanted islets, mass attrition can contribute to the overall function of the graft, since patients are followed for years after implant. The occurrence of β-cell attrition may well represent a key mechanism of graft failure, together with the potential metabolic effects of chronic immunosuppressive treatment and immune-mediated islet loss.

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