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ORIGINAL ARTICLE

Rapamycin Inhibits Growth Factor-Induced Cell Cycle Regulation in Pancreatic β Cells

Amy Aronovitz, MD,* Jami Josefson, MD,† Amanda Fisher, BS,* Marsha Newman, MS,*
Elizabeth Hughes, MS,* Fei Chen, BS,* David S. Moons, MD, PhD,‡
Hiroaki Kiyokawa, MD, PhD,§‡ and William L. Lowe, Jr, MD*

ABSTRACT

A progressive decline in islet function is a major obstacle to the success of islet transplantation. The cause of this decline in islet function is unclear, but immunosuppressive agents may contribute. Insulin-like growth factor-I (IGF-I) and betacellulin are important for islet cell survival and/or proliferation. In the present study, we performed studies in INS-1 cells and murine islets to define the effect of IGF-I and betacellulin on progression of islet cells through the cell cycle and the impact of immunosuppressive agents. Treatment of INS-1 cells for 24 hours with 20 ng/mL betacellulin or 50 ng/mL IGF-I increased cells in S phase by ~2-fold. Treatment of INS-1 cells with IGF-I or betacellulin also increased cyclin D1 expression and nuclear exclusion of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. In INS-1 cells and islets, betacellulin- and IGF-I increased Akt, extracellular signal-related kinase, and $p70^{S6\ kinase}$ phosphorylation. Rapamycin, an immunosuppressant which inhibits mammalian target of rapamycin, inhibited the increase in $p70^{S6\ kinase}$ phosphorylation stimulated by betacellulin- and IGF-I in INS-1 cells. Rapamycin also inhibited betacellulin- and IGF-I-induced entry of cells into S phase and 5′-Bromo-2′-deoxyuridine incorporation as well as the effect of betacellulin and IGF-I on cyclin D1 expression and nuclear exclusion of $p21^{Cip1}$ and $p27^{Kip1}$. Together, these data suggest that the effect of betacellulin and IGF-I on islet cell growth and proliferation is mediated, in part, via signaling through mammalian target of rapamycin. As rapamycin is used to treat islet transplant recipients, these results suggest that rapamycin could have deleterious effects on islet proliferation and function over time.

Key Words: betacellulin, insulin-like growth factor-I, islet, rapamycin

INTRODUCTION

Islet cell transplantation offers a potential cure for type 1 diabetes.[1] With the advent of the Edmonton protocol, euglycemia can be reliably achieved in islet transplant recipients,[2,3] although long-term follow-up studies have demonstrated a progressive decline of islet function over time.[4] The underlying mechanism for this functional decline is unclear, although it is not clearly related to allo- or autoimmune destruction of the islets. One possibility is that the immunosuppressive agents needed to prevent immune rejection of the islets contribute to the functional decline because of toxic effects on the islets.[5] Beyond the problem of functional decline over time, additional obstacles hinder the broad applicability of cell replacement therapy for diabetes. Among these is the requirement in most cases for islets from more than 1 pancreas to achieve euglycemia. A factor contributing to this need is the loss of transplant islet mass in the early posttransplant period.

The insulin-like growth factor (IGF) family, which includes IGF-I and -II, is important for modulating β-cell mass,[6,7] although the effects of the IGFs on β-cell mass are complex. The IGFs likely both inhibit β-cell apoptosis and modulate β-cell proliferation.[6–8] It has been shown previously that, in vitro, IGF-I protects pancreatic β-cells and islets in primary culture from cytokine-mediated apoptosis[9] and growth factor deprivation-induced cell death.[10] Insulin-like growth factor-I signals through a member of the tyrosine kinase family of transmembrane receptors, and its effect on cell survival is mediated, in

From the *Department of Medicine, Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Feinberg School of Med; †Department of Pediatrics, Children's Memorial Hospital and Northwestern University Feinberg School of Medicine; ‡Department of Biochemistry and Molecular Genetics, University of Illinois College of Medicine; and §Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Feinberg School of Medicine, Chicago, IL.

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Amy Aronovitz and Jami Josefson contributed equally to this work.

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Reprints: William L. Lowe, Jr, MD, Center for Endocrinology, Metabolism, and Molecular Medicine, Tarry 15-703, Northwestern University Feinberg School of Medicine, 303 East Chicago Ave., Chicago, IL 60611. E-mail: wlowe@northwestern.edu.

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part, by activation of the phosphatidylinositol (PI) 3-kinase pathway and its downstream target proteins, for example, Akt/protein kinase B.[11,12] The effect of IGF-I on pancreatic β cell proliferation is mediated by both the mitogen-activated protein kinase and the PI 3-kinase pathways, although details of its mechanism of action have not been fully elucidated.[8]

Betacellulin is a 32-kDa glycosylated protein that is a member of the epidermal growth factor family of peptides and was originally isolated from a mouse pancreatic tumor β cell line.[13] It is expressed in adult and fetal pancreas, signals through the family of tyrosine kinase receptors, and stimulates the proliferation of multiple cell types, including β-cells.[13,14] Different lines of evidence suggest that betacellulin plays a key role in islet cell proliferation. For one, betacellulin enhances pancreatic regeneration after 90% pancreatectomy by increasing β-cell proliferation and mass.[15] Second, it increases DNA synthesis in human fetal pancreatic epithelial cells and enhances β-cell development in fetal murine pancreatic explant cultures.[14,16] In vivo studies have shown increased β-cell mass and improved glucose tolerance after treatment with betacellulin.[15] Finally, in vitro studies have demonstrated that betacellulin treatment has a proliferative effect on insulinoma cell lines and rat and fetal human islets.[13,17–19] Like IGF-I, signaling through the ErbB family of receptors activates the extracellular signal-regulated kinase (ERK) and PI 3-kinase pathways.[8,20] To date, however, the mechanisms by which betacellulin mediates islet cell proliferation have not been fully elucidated.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase which is activated downstream of Akt in the PI 3-kinase pathway, and, thus, is activated by signaling through the IGF-I receptor as well as the ErbB family of receptors.[21–23] Indeed, previous studies have suggested that mTOR activation contributes to cell proliferation induced by growth factor signaling through these receptors.[21,24] As implied by its name, mTOR is inhibited by the immunosuppressive agent rapamycin, which is used to prevent graft rejection in islet transplant recipients. Given the above, the present study addressed the hypothesis that rapamycin inhibits growth factor-induced β-cell proliferation. In these studies, the effect of IGF-I and betacellulin on islet cell cycle progression, the signaling pathway(s) that mediate their effects, and the impact of rapamycin on their proliferative effects were examined. Studies were performed in INS-1 cells, a glucose-sensitive pancreatic β-cell line, and murine islets in primary culture. As growth factors may have beneficial effects on islets in the posttransplant period,[25] an effect of rapamycin on islet cell proliferation could explain, in part, its deleterious effects on islets. Thus, it is important to further define the impact of rapamycin on growth factor action in islet cells, with IGF-I and betacellulin being the 2 growth factors with important actions in islets.

■ MATERIALS AND METHODS

Cell Culture

The glucose-sensitive rat pancreatic β-cell line, INS-1, were obtained from Dr. Claes Wollheim (University Medical Center, Switzerland). The cells were maintained in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and incubated at 37°C in a 5% carbon dioxide and 95% air atmosphere.[26] Cells were subcultured at 70% to 80% confluence.

Islet Isolation

Islets from C57BL/6 male mice were isolated as previously described.[27,28] Mice were anesthetized with an intraperitoneal injection of 250 mg/kg tribromoethanol (Avertin; Sigma-Aldrich Company, St. Louis, MO). After a midline abdominal incision, the common bile duct was cannulated and injected with a cold solution of collagenase (type XI; Sigma-Aldrich) in Hanks balanced salt solution. The pancreas was dissected, removed, and digested at 37°C for 15 minutes. After filtration through a mesh screen, the filtrate was applied to a discontinuous dextran (Sigma-Aldrich) gradient. Islets were hand picked and counted under microscopic guidance and placed in RPMI 1640 media supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All studies were approved by the Northwestern University Animal Care and Use Committee.

Western Blot Analysis

Antibodies directed against phospho-ERK1/2, ERK, phospho-Akt, Akt, phospho-p70$^{S6\ kinase}$, p70$^{S6\ kinase}$, phospho-Fox01 (Ser^{256}), Fox01, phospho-glycogen synthase kinase 3β (Ser^{9}), and glycogen synthase kinase 3β (Cell Signaling Technology, Beverly, MA) were used at a dilution of 1:1000. For experiments performed with cell lysates from islets, phospho-p70$^{S6\ kinase}$ was used at a dilution of 1:500. Antibodies directed against cyclin D1 and cyclin D3 (Cell Signaling Technology) were used at a dilution of 1:2000, anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) at a dilution of 1:4000, and anti-α-tubulin (Sigma-Aldrich) at a dilution of 1:5000.

Western blot analyses were performed as described previously.[29] Briefly, INS-1 cells were plated at a density of 1.5×10^6 cells/60 mm dish. When cells reached ~70% confluence, they were washed with phosphate-buffered saline (PBS), and the medium was changed

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to RPMI 1640 with 1% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine for 16 to 24 hours before the treatment with 50 ng/mL IGF-I or 20 ng/mL betacellulin. Control cells were preincubated in RPMI 1640 with 1% FCS for the same period, and then treated with RPMI 1640 with 1% FCS without growth factors. For studies performed with the different inhibitors, rapamycin (EMD Biosciences, San Diego, CA), LY294002 (EMD Biosciences), and UO126 (Sigma-Aldrich), cells were treated without or with the indicated concentration of the inhibitor for 30 minutes before the treatment with IGF-I or betacellulin. After the treatment with the different growth factors, cell lysates were prepared in RIPA cell lysis buffer, and the protein content of the lysate was determined using the Coomassie blue protein assay following the manufacturer's instructions (Bio-Rad, Hercules, CA).

For studies with murine islets, 200 islets per treatment condition were serum-starved in RPMI 1640 with 1% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine for 2 hours before treatment with 100 ng/mL IGF-I or 20 ng/mL betacellulin. Control islets were treated in an identical manner but were not treated with IGF-I or betacellulin. After treatment with the growth factors, the islets were washed with cold PBS, and RIPA buffer was used to affect cell lysis using a Kontes hand-held homogenizer (Kimble/Kontes; Vineland, NJ). Protein content of the lysates was quantified as described previously.

For Western blot analyses, 20 μg of cell lysate were size separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) in a semi-dry apparatus. Membranes were blocked for 1 hour in TBS-T (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20) and 5% nonfat dry milk at room temperature. Membranes were incubated overnight at 4°C with primary antibodies with either 5% bovine serum albumin (BSA) or 5% nonfat dry milk. Membranes were then washed 3 times for 10 minutes in TBS-T, and secondary antibody (1:4000 dilution) was applied for 1 hour in TBS-T + 5% nonfat dry milk. The secondary antibody was either antihorseradish peroxidase-conjugated goat antirabbit IgG or antimouse IgG. After hybridization with the secondary antibody, the membranes were washed 3 times for 10 minutes in TBS-T. Immunoreactive bands were detected using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech (Arlington Heights, IL) according to the manufacturer's instructions.

To document equivalent loading of protein, the immunoblots were stripped in 62.5 mM Tris-HCl (pH 6.7), 20% SDS, and 100 mM β-mercaptoethanol for 30 minutes at 50°C. The membranes were then hybridized with the appropriate primary and secondary antibodies as described previously.

Flow Cytometry

Studies of cell cycle progression were performed using flow cytometry. For these experiments, INS-1 cells were plated at a density of 3.5×10^6 cells/100 mm plates. The cells were serum-starved in RPMI + 1% FCS media for 24 hours before the treatment. After the treatment without (control cells) or with either 50 ng/mL IGF-I or 20 ng/mL betacellulin for 6, 24, or 48 hours, cells were harvested and fixed in 1 mL of 70% cold ethanol at −20°C for a minimum of 2 hours. After fixation, the cells were washed 2 times in PBS and stained with 0.05 mg/mL propidium iodide solution at 37°C for 20 minutes in the dark. Samples were analyzed using a Beckman Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc., Miami, FL). The percentage of cells in different stages of the cell cycle was determined using the ModFit LT data fitting algorithm software (Verity Software Base, Topsham, ME).

5′-Bromo-2′-deoxyuridine (BrdU) Labeling of Cells

INS-1 cell proliferation was quantified using BrdU labeling. INS-1 cells were plated on glass coverslips, and upon reaching ~70% confluence, the cells were washed with PBS, and the media was changed to RPMI 1640 with 1% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine for 24 hours before treatment without (control cells) or with 50 ng/mL IGF-I or 20 ng/mL betacellulin for 24 hours. During the final 45 minutes of IGF-I or betacellulin treatment, 5 μg/mL of BrdU was added. The cells were then washed in PBS and fixed in 70% cold ethanol for 30 minutes. After fixation, the cells were treated with 2M HCl/0.5% Triton X-100 for 30 minutes followed by neutralization with 0.1 M sodium borate, pH 8.5. After two 3-minute washes in PBS, BrdU was detected using a 1:150 dilution of anti-BrdU antibody (Roche, Indianapolis IN) in 0.5% BSA and PBS and incubation for ~18 hours at 22°C in a humidified chamber. The cells were then washed twice for 3 minutes with cold PBS, and treated for 30 minutes at 22°C in the dark with fluorescein isothiocyanate-conjugated antimouse IgG (1:150 dilution; Invitrogen, Carlsbad, CA) in PBS + 0.5% BSA. The cells were washed twice again with cold PBS for 3 minutes. The cover slips were mounted using VECTASHIELD mounting medium with 4′.6-diamidino-2-phenylindole (DAPI) and stored in the dark at 4°C. To determine the impact of the different treatments on BrdU incorporation, a minimum of 300 cells per treatment condition were counted for each experiment. The percentage of $BrdU^+$ cells was determined by dividing the number of cells positive for BrdU staining by the total number of $DAPI^+$ cells.

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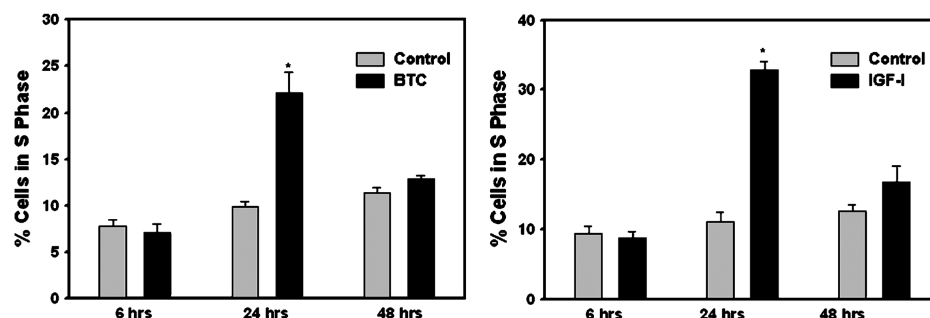

FIGURE 1. Effect of betacellulin and IGF-I on INS-1 cell cycle progression. Cells were treated with 20 ng/mL betacellulin (left panel) or 50 ng/mL IGF-1 (right panel) or, as a control, 1% FCS for 6, 24, and 48 hours. Cells were harvested at the indicated time points and analyzed by flow cytometry as described in Materials and Methods. Values represent the percentage of cells in S phase and are the mean ± SEM of 3 independent experiments. *$P < 0.01$ compared with control at 24 hours.

Immunohistochemical Analysis of $p21^{Cip1}$ and $p27^{Kip1}$

For studies on the cellular localization of $p21^{Cip1}$ and $p27^{Kip1}$, INS-1 cells were plated on glass coverslips, and upon reaching ~70% confluence, the cells were washed with PBS, and the media was changed to RPMI 1640 with 1% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine for 24 hours before treatment without (control) or with 50 ng/mL IGF-I or 20 ng/mL betacellulin for 6 hours. The cells were then washed in PBS and fixed in 3.7% paraformaldehyde for 20 minutes at room temperature. After fixation, the cells were treated with 0.1% Triton X-100 for 10 minutes at room temperature. The slides were blocked for 1 hour in goat serum followed by incubation overnight at 4°C with a 1:100 dilution of anti-$p21^{Cip1}$ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or 1:50 dilution of anti-$p27^{Kip1}$ antibody (Sigma-Aldrich). The cells were then washed 3 times in PBS and incubated for 30 minutes at room temperature with a 1:100 dilution of fluorescein anti-rabbit or antimouse antibody (Vector Laboratories, Burlingame, CA). The cells were washed twice again 3 times with PBS. The cover slips were mounted using VECTASHIELD mounting medium with DAPI and stored in the dark at 4°C. To determine the impact of the different treatments on cellular localization, a total of at least 300 cells per treatment condition were counted for each experiment. The percentage of cells with nuclear localization of p21 or p27 was determined by dividing the number of cells with p21 or p27 localized to the nucleus by the total number of $DAPI^+$ cells.

Statistical Analyses

Values are reported at the mean ± SEM. SigmaStat 9.0 software (Systat Software, San Jose, CA) was used for all statistical analyses. *P* values were calculated using analysis of variance with the Fisher protected least significant difference for multiple comparisons, the Holm-Sidak method for pairwise comparison, or Student *t* test where $P < 0.05$ was considered statistically significant.

RESULTS

Effect of IGF-I and Betacellulin on Cell Cycle Progression in INS-1 Cells

Flow cytometry was used to define the impact of IGF-I and betacellulin treatment on cell cycle progression in INS-1 cells. Cells were treated with 50 ng/mL IGF-I or 20 ng/mL betacellulin for 6, 24, or 48 hours and analyzed by flow cytometry. As shown in Fig. 1, after 6 hours of treatment with IGF-I or betacellulin, there was no change in the percentage of cells in S phase compared with control cells. However, after 24 hours, the percentage of cells in S phase increased significantly in both the IGF-I- and betacellulin-treated cells compared with control cells ($P < 0.001$). By 48 hours, the percentage of cells in S phase had decreased toward control levels. These results demonstrate that IGF-I and betacellulin treatment enhance INS-1 cell progression through the cell cycle.

IGF-I- and Betacellulin-Induced Signaling in INS-1 Cells and Murine Islets

Subsequent studies were performed to begin to define the molecular mechanism for the effect of IGF-I and betacellulin on INS-1 cell cycle progression. These growth factors activate the ERK and PI 3-kinase pathways,[8,20] so initial studies examined the ability of IGF-I and betacellulin to activate these 2 signaling pathways in INS-1 cells as well as murine islets in primary culture. INS-1 cells were treated for varying periods with either 50 ng/mL IGF-I or 20 ng/mL betacellulin (Fig. 2A). Insulin-like growth factor-I- and betacellulin-induced ERK phosphorylation was transient with a maximal increase in ERK phosphorylation observed after 15 minutes of IGF-I treatment and 10 minutes of betacellulin treatment. Akt is a kinase which is downstream of PI 3-kinase and important in mediating the effects of IGF-I and other growth factors on cell survival.[22] Both IGF-I and betacellulin also induced Akt phosphorylation, although, in contrast to ERK, Akt phosphorylation was sustained at for least 60 to 120 minutes after ligand treatment.

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Betacellulin treatment also increased the phosphorylation 2 additional downstream targets of the PI 3-kinase pathway, Fox01, a member of the forkhead family of transcription factors which is known to impact cell cycle progression, and glycogen synthase kinase-3β, a kinase which induces apoptosis and is inhibited by phosphorylation (data not shown).

To confirm that IGF-I and betacellulin were capable of activating these same pathways in pancreatic islets, the ability of IGF-I and betacellulin to stimulate phosphorylation of these kinases in murine islets was examined (Fig. 2B). Purified islets were treated for 15 and 30 minutes with either 100 ng/mL IGF-I or 20 ng/mL betacellulin. As in INS-1 cells, IGF-I and betacellulin increased both ERK and Akt phosphorylation.

Through its effect on the tuberous sclerosis complex 2 and proline-rich Akt substrate of 40 kDa, Akt indirectly activates the mTOR.[22] Mammalian target of rapamycin is important in regulating cell growth and metabolism,[23] and its effects are mediated, in part, by activation of $p70^{S6\ kinase}$.[23] To determine whether IGF-I and betacellulin activate the mTOR pathway in INS-1 cells and murine islets, the effect of IGF-I and betacellulin treatment on the phosphorylation of $p70^{S6\ kinase}$ was examined (Fig. 2C). Treatment of INS-1 cells with either 50 ng/mL IGF-I or 20 ng/mL betacellulin increased $p70^{S6\ kinase}$ phosphorylation. Insulin-like growth factor-I treatment resulted in a sustained increase in $p70^{S6\ kinase}$ phosphorylation, whereas betacellulin treatment stimulated a transitory increase in $p70^{S6\ kinase}$ phosphorylation with a peak effect

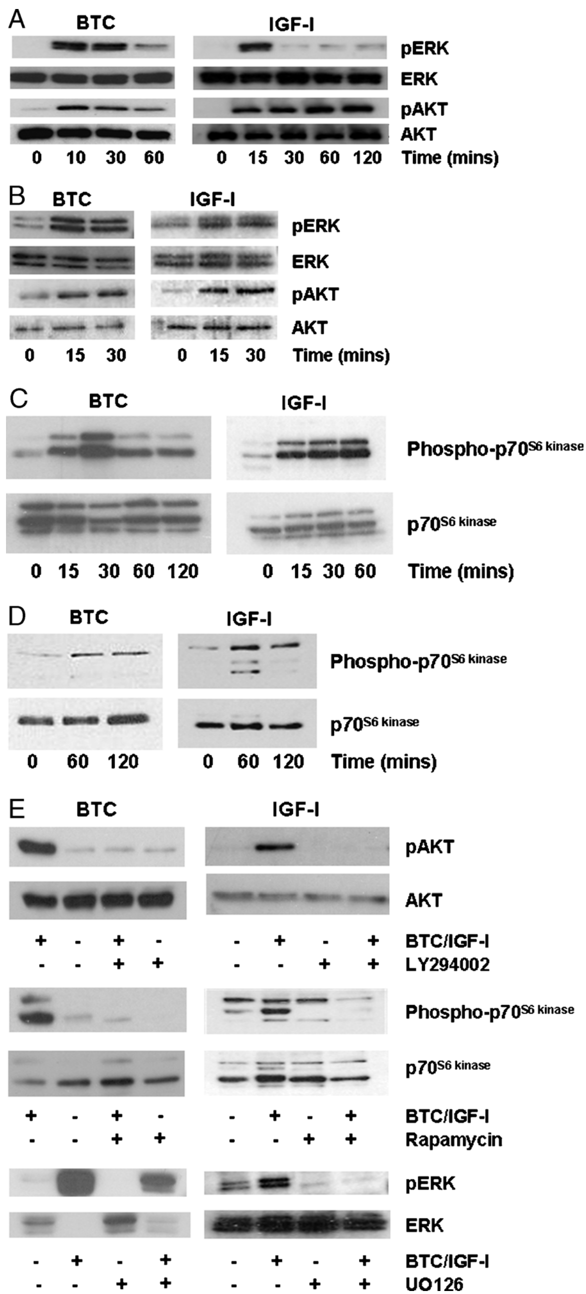

FIGURE 2. Time course of betacellulin- and IGF-I-induced signaling in INS-1 cells and murine islets. Panel A, INS-1 cells were treated for the indicated periods with either 20 ng/mL betacellulin (BTC) or 50 ng/mL IGF-I. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against phospho-ERK and phospho-Akt as described in Materials and Methods. The blots were then stripped and reprobed with antibody directed against either ERK or Akt. The results are representative of 3 independent experiments performed using different cell lysates. Panel B, Time course of betacellulin- and IGF-I-induced signaling in murine islets. After isolation, murine islets were treated for the indicated period with either 20 ng/mL betacellulin or 100 ng/mL IGF-I. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against phospo-ERK, ERK, phospho-Akt, and Akt as described previously and in Materials and Methods. The results are representative of 2 independent experiments performed using different isolations of murine islets. Panel C, betacellulin- and IGF-I-induced $p70^{S6\ kinase}$ phosphorylation in INS-1 cells. INS-1 cells were treated for the indicated period with either 20 ng/mL betacellulin or 50 ng/mL IGF-I, and Western blot analyses were performed using antibodies directed against anti-phospho-$p70^{S6\ kinase}$ and anti-$p70^{S6\ kinase}$ as described previously. Panel D, betacellulin- and IGF-I-induced $p70^{S6\ kinase}$ phosphorylation in murine islets. Murine islets were treated for the indicated period with either 20 ng/mL betacellulin or 100 ng/mL IGF-I, and Western blot analyses were performed using antibodies directed against phospho-$p70^{S6\ kinase}$ and $p70^{S6\ kinase}$ as described previously. Panel E, effect of inhibitors on growth factor-induced signaling in INS-1 cells. INS-1 cells were pretreated for 30 minutes with either 20 μM LY294002, 20 μM U0126, or 25 nM rapamycin and then treated for 15 minutes with either 20 ng/mL betacellulin or 50 ng/mL IGF-I in the absence or presence of 1 of the inhibitors. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against phospho-ERK, ERK, phospho-Akt, Akt, phospho-$p70^{S6\ kinase}$ and $p70^{S6\ kinase}$ as described previously and in Materials and Methods.

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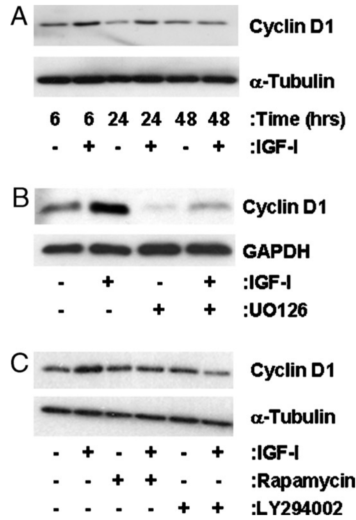

FIGURE 3. Panel A, effect of IGF-I on cyclin D1 levels in INS-1 cells. INS-1 cells were incubated in the absence or presence of 50 ng/mL IGF-I for the indicated periods. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against cyclin D1 as described Materials and Methods. The blots were stripped and reprobed with an antibody directed against α-tubulin. Panel B, effect of UO126 on IGF-I-induced cyclin D1 expression. INS-1 cells were pretreated for 30 minutes with 20 μM UO126 and then treated for 6 hours without or with 50 ng/mL IGF-I in the absence or presence of UO126. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against cyclin D1 or GAPDH for the stripped blot as described previously. Panel C, effect of LY294002 and rapamycin on IGF-I-induced cyclin D1 expression. INS-1 cells were pretreated for 30 minutes with 20 μM LY294002 or 25 nM rapamycin, and then treated for 6 hours without or with 50 ng/mL IGF-I in the absence or presence of LY294002 or rapamycin. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against either cyclin D1 or α-tubulin as described previously. The results in Panels A to C are representative of 2 independent experiments performed using different INS-1 cell lysates.

observed after 30 minutes of betacellulin treatment. The impact of IGF-I and betacellulin treatment on $p70^{S6\ kinase}$ phosphorylation in murine islets was also examined (Fig. 2D). In islets, the effect of IGF-I and betacellulin was somewhat delayed compared with INS-1 cells, but after the treatment of islets with 100 ng/mL IGF-I or 20 ng/mL betacellulin for 60 or 120 minutes, increased $p70^{S6\ kinase}$ phosphorylation was evident with a peak effect at 60 minutes.

To document the efficacy of different inhibitors of IGF-I- and betacellulin-induced signaling in INS-1 cells, studies were undertaken with LY294002, an inhibitor of Akt; rapamycin, an inhibitor of mTOR; and UO126, an inhibitor of ERK. Treatment with 20 μM LY294002 inhibited IGF-I- and betacellulin-induced Akt phosphorylation (Fig. 2E, top panel), and, as expected, rapamycin, which inhibits signaling downstream of Akt, had no effect on IGF-I-induced Akt phosphorylation (data not shown). In cells treated for 15 minutes with 50 ng/mL IGF-I or 20 ng/mL betacellulin, 25 nM rapamycin, markedly

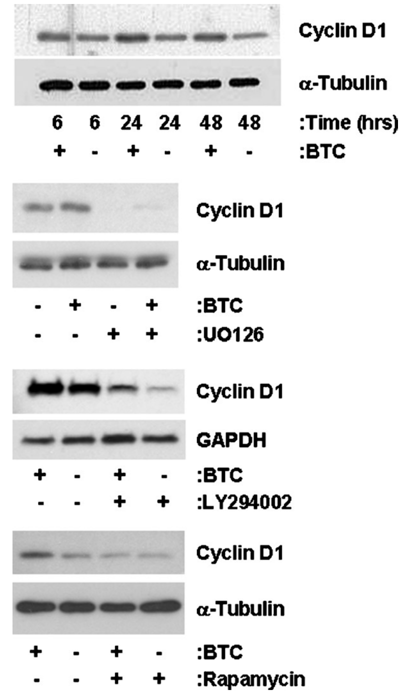

FIGURE 4. Panel A, effect of betacellulin on cyclin D1 levels in INS-1 cells. INS-1 cells were incubated in the absence or presence of 20 ng/mL betacellulin for the indicated periods. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against cyclin D1 as described in Materials and Methods. The blots were stripped and reprobed with an antibody directed against α-tubulin. Panel B, effect of UO126 on betacellulin-induced cyclin D1 expression. INS-1 cells were pretreated for 30 minutes with 20 μM UO126 and then treated for 6 hours without or with 20 ng/mL betacellulin in the absence or presence of UO126. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against either cyclin D1 or α-tubulin as described previously. Panel C, effect of LY294002 on betacellulin-induced cyclin D1 expression. INS-1 cells were pretreated for 30 minutes with 20 μM LY294002, and then treated for 6 hours without or with 20 ng/mL betacellulin in the absence or presence of LY294002. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against cyclin D1 or GAPDH for the stripped blot as described previously. Panel D, effect of rapamycin on betacellulin-induced cyclin D1 expression. INS-1 cells were pretreated for 30 minutes with 25 nM rapamycin, and then treated for 6 hours without or with 20 ng/mL betacellulin in the absence or presence of rapamycin. Cell lysates were prepared, and Western blot analyses were performed using antibodies directed against either cyclin D1 or α-tubulin as described previously. The results in Panels A to D are representative of 2 independent experiments performed using different INS-1 cell lysates.

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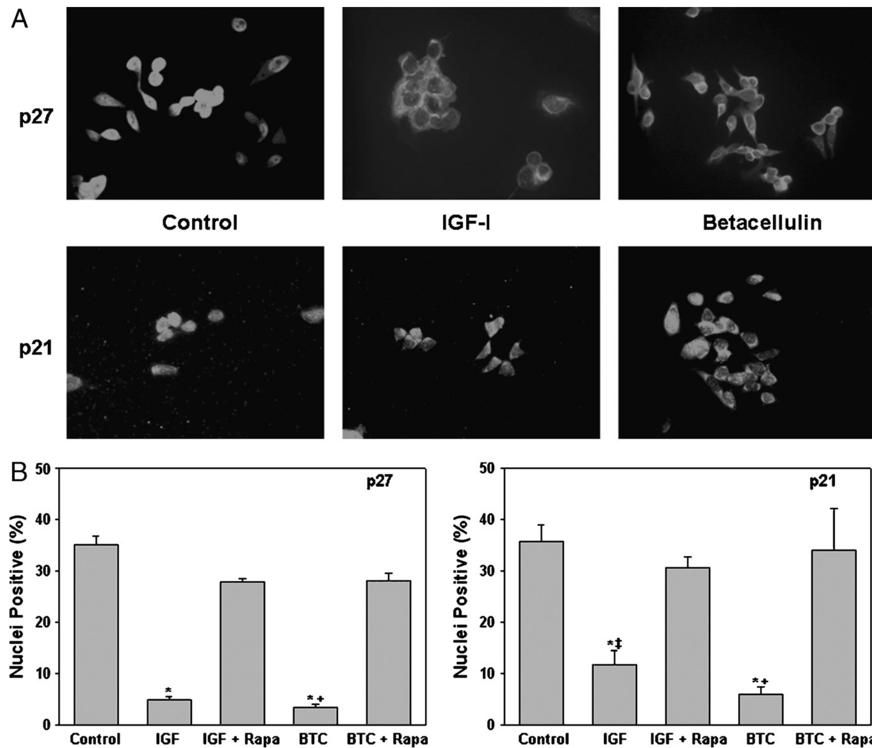

FIGURE 5. Effect of betacellulin and IGF-I on the cellular localization of $p21^{Cip1}$ and $p27^{Kip1}$. Panel A, INS-1 cells were treated for 6 hours with either 20 ng/mL betacellulin or 50 ng/mL IGF-I. The cells were then fixed, and immunohistochemical analyses were performed using antibodies directed against $p21^{Cip1}$ and $p27^{Kip1}$ as described in Materials and Methods. 40× magnification. Panel B, effect of rapamycin on betacellulin- and IGF-I-induced changes in cellular localization of $p27^{Kip1}$ (left panel) and $p21^{Cip1}$ (right panel). INS-1 cells were pretreated without or with 25 nM rapamycin, and then treated for 6 hours with 20 ng/mL betacellulin or 50 ng/mL IGF-I in the absence or presence of the inhibitor. Immunohistochemical analyses were performed as described previously, and more than 300 cells were counted for each treatment condition for each experiment. The results are the percentage of cells in which $p21^{Cip1}$ or $p27^{Kip1}$ were localized in the nucleus. The values are the mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ compared with control. $^{+}P < 0.05$ compared with BTC + Rapa. $^{\ddagger}P < 0.05$ compared with IGF + Rapa.

reduced $p70^{S6\ kinase}$ phosphorylation (Fig. 2E, middle panel). Finally, 20 μM UO126 effectively inhibited ERK phosphorylation in INS-1 cells treated with 50 ng/mL IGF-I for 15 minutes (Fig. 2E). UO126 was also effective in inhibiting the effect of 20 ng/mL betacellulin on ERK phosphorylation, although the inhibition was only partial (Fig. 2E, bottom panel).

Effect of IGF-I on Cyclin D1 Expression

Cyclin D1 regulates cyclin-dependent kinase activity, and its expression is important for cell cycle progression.[30] To determine whether IGF-I and betacellulin enhance cyclin D1 expression, INS-1 cells were treated with either 50 ng/mL IGF-I or 20 ng/mL betacellulin for 6, 24, and 48 hours (Figs. 3 and 4). Insulin-like growth factor-I treatment increased cyclin D1 levels at 6 and 24 hours with a return to baseline levels by 48 hours. In betacellulin-treated cells, cyclin D1 levels were increased at all time points. Betacellulin also increased cyclin D3 levels after 24 and 48 hours of treatment (data not shown).

To define the signaling pathway that was important for mediating the effect of IGF-I and betacellulin on cyclin D1 levels, the impact of the different inhibitors on IGF-I- and betacellulin-induced cyclin D1 expression was examined. In INS-1 cells treated for 6 hours with 50 ng/mL IGF-I, 20 ng/mL betacellulin, treatment with 20 μM of either LY294002 or U0126 inhibited the IGF-I- and betacellulin-induced increase in cyclin D1 expression (Figs. 3 and 4), suggesting that signaling through both the ERK and PI 3-kinase pathways contribute to growth factor-induced cyclin D1 expression in islet cells.

As noted, $70^{S6\ kinase}$ is phosphorylated by the PI 3-kinase pathway via activation of mTOR.[22,23] Given the use of rapamycin, an inhibitor of mTOR, as an immunosuppressive agent in islet transplantation, further studies were performed to examine its impact on the IGF-I- and betacellulin-induced increase in cyclin D1 levels. Treatment with 25 nM rapamycin abrogated the IGF-I- and betacellulin-induced increase in cyclin D1 levels (Figs. 3 and 4).

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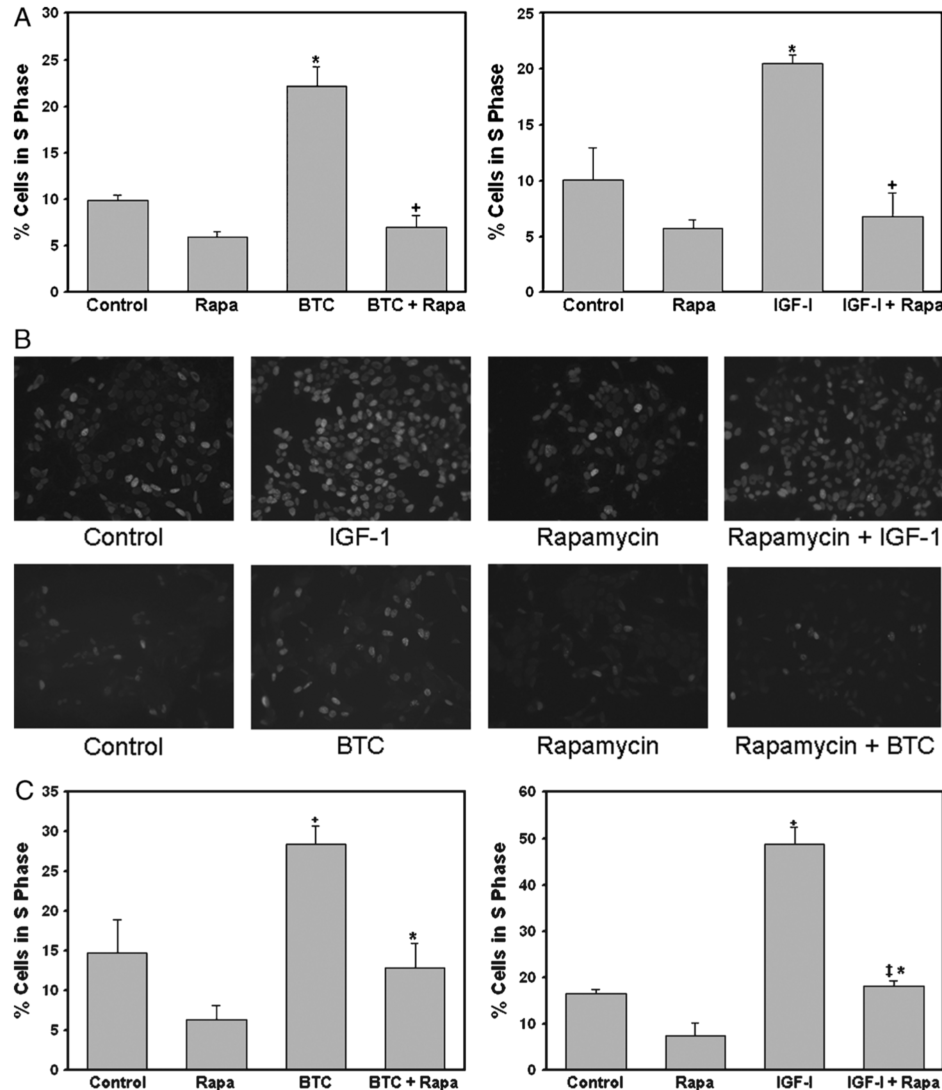

FIGURE 6. Panel A, effect of Rapamycin on betacellulin- and IGF-1-induced cell cycle progression in INS-1 cells. INS-1 cells were pretreated for 30 minutes with 25 nM Rapamycin and treated for 24 hours with either 20 ng/mL betacellulin (left panel), 50 ng/mL IGF-1 (right panel), or 1% FCS (control) in the absence or presence of rapamycin. The cells were then analyzed by flow cytometry as described in Materials and Methods. Values represent the percentage of cells in S phase and are the mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ compared with control. $^{+}P < 0.05$ compared with BTC or IGF-I cells. Panel B, inhibition by rapamycin of betacellulin- and IGF-I-induced BrdU incorporation into INS-1 cells. After the pretreatment for 30 minutes with 25 nM rapamycin, INS-1 cells were treated for 24 hours with either 20 ng/mL betacellulin, 50 ng/mL IGF-I, or 1% FCS in the absence or presence of 25 nM rapamycin. During the final 45 minutes of IGF-I or betacellulin treatment, 5 μg/mL of BrdU was added. The cells were then fixed and analyzed using an antibody directed against BrdU (green) and stained with DAPI (blue) as described in Materials and Methods. 20× magnification. Panel C, quantification of impact of rapamycin on betacellulin- and IGF-I-induced BrdU incorporation. INS-1 cells were treated as described previously, and more than 300 cells were counted for each treatment condition for each experiment. The values represent the percentage of cells with $BrdU^{+}$ nuclei and are the mean ± SEM of 3 independent experiments. $^{+}P < 0.05$ compared with control. $^{*}P < 0.05$ compared with BTC or IGF-I. $^{\ddagger}P < 0.05$ compared with Rapa.

$p21^{Cip1}$ and $p27^{Kip1}$ are members of the family of cyclin-dependent kinase inhibitors which appear to play a role in limiting the proliferation of β-cells.[31,32] INS-1 cells treated for 6, 24, and 48 hours with 50 ng/mL IGF-I or 20 ng/mL betacellulin did not exhibit a consistent effect on the level of $p21^{Cip1}$ and $p27^{Kip1}$ (data not shown). Subsequent studies determined whether IGF-I or betacellulin affected the cellular localization of $p21^{Cip1}$ and $p27^{Kip1}$ (Figs. 5A and B). Treatment of INS-1 cells for 6 hours with either 50 ng/mL IGF-I or 20 ng/mL betacellulin markedly decreased the percentage of nuclei which were positive for $p21^{Cip1}$ and $p27^{Kip1}$. Interestingly, treatment with 25 nM

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rapamycin effectively inhibited the ability of IGF-I and betacellulin to stimulate transport p21^{Cip1} and p27^{Kip1} transport out of the nucleus (Fig. 5B).

Effect of Rapamycin on IGF-I- and Betacellulin-Induced Cell Cycle Progression and Proliferation of INS-1 Cells

Having demonstrated the impact of rapamycin on the expression and cellular localization of proteins important for cell cycle progression, studies were performed to examine the impact of rapamycin on IGF- and betacellulin-induced cell cycle progression and proliferation of INS-1 cells. In INS-1 cells treated for 24 hours with either 50 ng/mL IGF-I or 20 ng/mL betacellulin in the absence or presence of 25 nM rapamycin, IGF-I and betacellulin significantly increased the percentage of cells in S phase, and this effect of IGF-I and betacellulin was inhibited by rapamycin (Fig. 6).

5′-Bromo-2′-deoxyuridine labeling was used to further examine the impact of rapamycin on IGF-I- and betacellulin-induced INS-1 cell proliferation. After the treatment of INS-1 cells for 24 hours with 50 ng/mL IGF-I or 20 ng/mL betacellulin, IGF-I and betacellulin enhanced INS-1 cell proliferation as reflected by a greater than 2-fold increase in the percentage of BrdU^{+} cells in IGF-I- and betacellulin-treated versus control cells. In the presence of 25 nM rapamycin, IGF-I and betacellulin stimulated a small increase in the percentage of BrdU^{+} cells, although the magnitude of this increase was markedly reduced compared with the increase simulated by IGF-I and betacellulin in the absence of rapamycin. Previous studies suggest that signaling through mTOR contributes to IGF-I- and betacellulin-induced INS-1 cell cycle progression and proliferation.

■ DISCUSSION

Islet transplantation has been complicated by long term islet failure.[4] The mechanism for this remains unknown, but recent studies demonstrated that the combination of rapamycin and tacrolimus inhibited β-cell regeneration in a murine model of diabetes[33] and rapamycin inhibited pregnancy-induced proliferation of murine islet cells.[34] In the present study, we demonstrated the importance of signaling through mTOR, which is inhibited by rapamycin, for in vitro β-cell proliferation induced by 2 growth factors, IGF-I and betacellulin. These results may provide an explanation for the recent observations of Nir et al.[33] and Zahr et al.[34]

Members of the IGF and EGF families are known to stimulate progression through the cell cycle, but, to date, their role in cell cycle progression in pancreatic β-cells has not been well studied. To address that question, we used INS-1 cells, a pancreatic β-cell line, as a model system to demonstrate that treatment with either betacellulin or IGF-I increased the fraction of cells in S phase of the cell cycle and BrdU incorporation into DNA, suggesting that these growth factors can stimulate β-cell proliferation. Treatment of INS-1 cells with either betacellulin or IGF-I activated the ERK and PI 3-kinase pathways as well as p70$^{S6\ kinase}$, which is downstream of PI 3-kinase. Importantly, these same pathways were also activated by betacellulin and IGF-I in native murine islets. Subsequent studies with inhibitors of the different pathways suggested that signaling through mTOR was important for mediating the effect of these growth factors on cell cycle progression and proliferation.

Mammalian target of rapamycin is serine/threonine kinase which is a member of the PI 3-kinase-related kinase superfamily, a family of kinases which play an important role in the regulation of cell growth (ie, cell mass and size) as well as cell cycle checkpoint control.[35] Indeed, inhibition of signaling through mTOR has been shown to restrict cell cycle progression in an osteosarcoma cell line.[35] Mammalian target of rapamycin is activated by a variety of extracellular signals including growth factors like insulin and IGF-I.[23] Insulin-like growth factor-I-induced activation of mTOR and phosphorylation of 2 of its downstream targets, p70$^{S6\ kinase}$ and 4E-BP1, has been demonstrated previously in INS-1 cells,[21,36,37] and signaling through this pathway mediated, in part, IGF-I-induced stimulation of thymidine incorporation into INS-1 cells.[37] This pathway also stimulated the induction of proliferating cell nuclear antigen expression by insulin in an immortalized fetal pancreatic β-cell line.[38] To date, the role of mTOR in mediating the effects of betacellulin or signaling through members of the EGF receptor family, in general, has not been examined in β-cells. The results of the present study have now established that betacellulin stimulates signaling through the mTOR pathway in both INS-1 cells and native islets and have extended the results of previous studies by demonstrating that IGF-I stimulates p70$^{S6\ kinase}$ phosphorylation in native islets. Moreover, the present study has now demonstrated that signaling through mTOR mediates IGF-I- and betacellulin-stimulated cell cycle progression in INS-1 cells. Consistent with the role of this pathway in mediating this effect of IGF-I and betacellulin, signaling through mTOR also modulated the stimulatory effect of these growth factors on both cyclin D1 levels and the cellular localization of the cyclin-dependent kinase inhibitors, p21^{Cip1} and p27^{Kip1}.

A recent study demonstrated that mTOR contributes to glucose-stimulated thymidine incorporation into DNA and accumulation of pancreatic β-cells in S phase of the cell cycle.[39] However, to date, the impact of mTOR on cell cycle regulatory proteins in pancreatic β-cells has not been reported. We have now established that rapamycin inhibits the effect of IGF-I and betacellulin on cyclin

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D1 levels and cell cycle progression in pancreatic β-cells. This result is consistent with previous findings in prostate, ovarian, and breast cancer cell lines, vascular smooth muscle cells, and fibroblasts, where rapamycin is able to inhibit cell cycle progression in concert with a decrease in cyclin D1 levels.[40–44] Rapamycin's effect on cyclin D1 levels is important because in mammary tumor cells, overexpression of cyclin D1 partly overcomes rapamycin's inhibitory effect on cell cycle progression.[44] Cyclin D1 also plays a key role in promoting proliferation of β-cells by activating its catalytic partner, cyclin-dependent kinase 4 (CDK4). Our recent study demonstrated that siRNA-mediated knockdown of CDK4 inhibits proliferation of INS-1 cells (D.S. Moons and H. Kiyokawa, unpublished observations), consistent with the studies showing that postnatal *Cdk4*-deficient mice are defective in islet proliferation.[45,46] Interestingly, the inhibitory effect of rapamycin on cell cycle regulation is cell-type specific. In NIH 3T3 (M17) fibroblasts, activation of PI 3-kinase is required for EGF to increase cyclin D1 levels and promote cell cycle progression; however, rapamycin did not abrogate these effects of EGF.[47]

Beyond its effect on cyclin D1, signaling through the mTOR pathway may have also contributed to betacellulin- and IGF-I-induced cell cycle progression via an effect on the cellular localization of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1}$. $p27^{Kip1}$ is expressed in pancreatic islet cells and inhibits β-cell proliferation.[31,32,48] $p21^{Cip1}$ is also expressed in islets. Interestingly, some β-cell mitogens, including hepatocyte growth factor and placental lactogen, increase $p21^{Cip1}$ levels in islets.[32,49] Despite this, the effect of these growth factors on β-cell proliferation is augmented in $p21^{-/-}$ mice, suggesting that $p21^{Cip1}$ inhibits β-cell proliferation.[32,49] In the nucleus, $p27^{Kip1}$ and $p21^{Cip1}$ inhibit cell cycle progression by binding to complexes of the cyclins and cdk-2, -4, and -6. This interaction inhibits the kinase activity of the complex. However, in cytoplasm, $p27^{Kip1}$ and $p21^{Cip1}$ serve as molecular chaperones for the formation of cyclin-cdk complexes and facilitate their transport into the nucleus where they promote cell cycle progression.[31] We have now demonstrated that treatment with either betacellulin or IGF-I results in nuclear exclusion of $p27^{Kip1}$ and $p21^{Cip1}$. Cytoplasmic localization of $p27^{Kip1}$ and $p21^{Cip1}$ would prevent their binding to nuclear cyclin-cdk complexes with the net effect of increasing cdk kinase activity and promoting cell cycle progression. Like their effect on cyclin D1 levels, this effect of betacellulin and IGF-I is mediated via signaling through mTOR. The role of mTOR in nuclear export of $p27^{Kip1}$ has been examined previously in another endocrine cell type, thyrotropes, in which thyroid stimulating hormone in combination with serum stimulates nuclear export of $p27^{Kip1}$ in a rapamycin-inhibitable manner.[50] To date, the effect of betacellulin on cellular localization of $p27^{Kip1}$ and $p21^{Cip1}$ had not been previously studied. A recent study demonstrated that IGF-I stimulates the nuclear export of $p27^{Kip1}$ in pancreatic β-cells, although the signaling pathway mediating this effect of IGF-I was not defined.[51]

Previous studies have demonstrated an effect of rapamycin on insulin secretion as well as vascular endothelial growth factor production and secretion by pancreatic β-cells, β-cell apoptosis, and the function of islet transplant grafts.[52–57] Our study now shows that rapamycin can abrogate β-cell proliferation induced by growth factors which signal through transmembrane receptors with intrinsic tyrosine kinase activity. We demonstrated that rapamycin decreases the proportion of cells in S phase of the cell cycle and cell proliferation after the treatment with either IGF-I or betacellulin. These new findings are consistent with the recent report that rapamycin in combination with tacrolimus inhibited β-cell regeneration in a murine model of diabetes and could help to explain the progressive loss of islet function after islet transplantation. The cause of this failure is, at present, not clear but may be related, in part, to the negative impact of immunosuppressive agents, including rapamycin, on β-cell function. Although only 0.5% of pancreatic β-cells are undergoing mitosis at any one time in the basal state,[58] a previous study has demonstrated that islet mass is regulated in both physiological and pathophysiological states by a balance between cell proliferation and death.[59] Thus, by inhibiting or slowing islet cell proliferation, rapamycin may upset this balance between cell proliferation and death leading to a progressive loss of islet mass over time. Our data demonstrate that rapamycin-mediated inhibition of signaling through the mTOR pathway had several effects on β-cells which would decrease cell proliferation. This includes inhibiting both growth factor-induced accumulation of cyclin D1 and the translocation of cyclin-dependent kinase inhibitors from the nucleus to the cytoplasm.

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