Agonist of growth hormone-releasing hormone as a potential effector for survival and proliferation of pancreatic islets

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Therapeutic strategies for transplantation of pancreatic islet cells are urgently needed to expand β-cell mass by stimulating islet cell proliferation and/or prolonging islet cell survival. Control of the islets by different growth factors provides a potential venue for augmenting β-cell mass. In the present study, we show the expression of the biologically active splice variant-1 (SV-1) of growth hormone-releasing hormone (GHRH) receptor in rat islet cells (INS-1) and in rat and human pancreatic islets. In studies in vitro of INS-1 cells, the GHRH agonist JI-36 caused a significant increase in cell proliferation and a reduction of cell apoptosis. JI-36 increased islet size and glucose-stimulated insulin secretion in isolated rat islets after 48–72 h. At the ultrastructural level, INS-1 cells treated with agonist JI-36 revealed a metabolic active stimulation state with increased cytoplasm. Coincubation with the GHRH antagonist MIA-602 reversed the actions of the agonist JI-36, indicating the specificity of this agonist. In vivo, the function of pancreatic islets was assessed by transplantation of rat islets under the kidney capsule of streptozotocin-induced diabetic non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. Islets treated with GHRH agonist JI-36 were able to achieve normoglycemia earlier and more consistently than untreated islets. Furthermore, in contrast to diabetic animals transplanted with untreated islets, insulin response to an i.p. glucose tolerance test (IPGTT) in animals receiving islets treated with agonist JI-36 was comparable to that of normal healthy mice. In conclusion, our study provides evidence that agonists of GHRH represent a promising pharmacological therapy aimed at promoting islet graft growth and proliferation in diabetic patients.

diabetes | islet proliferation | regenerative therapies

Transplantation of pancreatic islet cells is a valid treatment option for selected patients with brittle diabetes. Under current protocols, the main therapeutic goal that can be reliably achieved is improved glycemic control and prevention of severe hypoglycemic episodes. Insulin independence can only be achieved for a limited time after repeated transplantations (1) due to insufficient islet mass and progressive loss of islets over time. Therefore, efforts to improve islet transplantation focus on improving the exploitation of mechanisms governing β-cell proliferation and growth as well as islet quality (2–4).

Several growth factors that may have potential for enhancing β-cell mass have been identified (5). A natural growth factor-mediated adaptation of islet cell mass occurs due to increased demand during pregnancy as well as with obesity (6). In addition, promotion of islet cell growth has been linked to glucagon-like peptide 1 (GLP-1), obestatin, and ghrelin (4, 7, 8). Surprisingly, little attention has been given to the possible role of growth hormone-releasing hormone (GHRH) or its agonists. In his Nobel lecture more than 60 y ago, Bernardo Houssay described the critical role of the “hypophysis in carbohydrate metabolism and in diabetes” (9). He observed that extracts of the anterior pituitary gland can produce a stimulation and hyperplasia of islets under certain conditions. With the advent of stem cell biology and regenerative medicine, there has now been a renewed interest in elucidating the role of hypothalamic-pituitary growth factors in islet cell regulation.

GHRH stimulates the release of growth hormone (GH) from the pituitary and has been the focus of intense studies since its structure was described in 1982 (10, 11). The full biological activity of GHRH resides in the N-terminal 1–29 amino acid sequence of this peptide (12). GHRH and the pituitary type of GHRH receptor as well as its splice variants are expressed in many human tissues (i.e., ovary, testis, pancreas, colon, esophagus, breast, kidney, liver, prostate, lungs, and thymus) (13–15).

Recent study has shown that rat GHRH promoted survival of cardiomyocytes in vitro and protected rat hearts from ischemia-reperfusion injury (16). The detection of the GHRH receptor (GHRH-R) on the cardiomyocyte sarcolemma supports the view that GHRH may elicit direct signal transduction within the heart, independent of the GH/IGF1 axis per se (17). Synthetic GHRH agonists, such as JI-36 (GHRH-A), are more potent and longer-acting than native GHRH (18, 19). Recently, we showed that GHRH-agonist JI-36 has a favorable cardiac effect, attenuating infarct size as well as the progressive decrease of cardiac structure and function following myocardial infarction (MI) (16).

Finally, GHRH has been shown to promote angiogenesis by increasing vascular endothelial growth factors (VEGF) (20). VEGF and vascularization play a crucial role in β-cell function and islet regeneration (21, 22). In the present study, we show expression of GHRH receptor splice variant-1 (SV-1) (23, 24) in rat insulinoma INS-1 cells as well as in rat and human pancreatic islets. We also analyzed the effect of a synthetic GHRH agonist on β-cell survival and cell proliferation in vitro and in vivo. In addition, we tested the effect of this agonist, JI-36, on β-cells before transplantation in a diabetic animal model.

Results

Expression of Receptor for GHRH in Insulinoma Cells and in Rat Islet Cells. RT-PCR analysis showed expression of GHRH receptor (564 bp) in INS-1 cells and in rat islets. Rat pituitary was used as a positive control (Fig. L4). In addition, the protein of the biologically more-active splice variant SV-1 of GHRH receptor


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was detected in INS-1 and rat islets by Western blotting (39.5 kDa). Rat pituitary was used as a positive control (Fig. 1B).

**Immunohistochemical Confirmation of the Expression of GHRH Receptor Protein in Insulinoma Cells and Rat and Human Islets.** Immunohistochemical analysis showed pronounced GHRH-R immunostaining of INS-1 cells (Fig. 2A and D), rat (Fig. 2B and E), and human (Fig. 2C and F) islets. To confirm the localization of GHRH-R on β-cells, costaining for insulin was performed (Fig. 2F).

**Ultrastructural Analysis of Insulinoma Cells Before and After Incubation with the GHRH Agonist.** INS-1 cells under normal culture conditions were characterized by secretory granules close to the cell membrane (Fig. 3A). The cell surface itself extended long filopodia and other membrane protrusions (Fig. 3B). Treatment of islet cells with 10^{-6} M JI-36 produced an enlargement of the cell membrane and the volume of the cytoplasm. This was accompanied by the disappearance of membrane protrusions (Fig. 3C). Furthermore, mitochondria and lysosomes were also enlarged; the latter contained numerous vesicles, indicating intracytoplasmatic digestion of the contents (peptides, proteins) of secretory vacuoles, after the vacuoles fuse with lysosomes. Additional changes became obvious in the cell nucleus, demonstrating an increased amount of heterochromatin as well as nucleoli (Fig. 3D). These morphological changes suggest an increased active metabolic state of the islet cells.

**Cell Proliferation Studies on Insulinoma Cells.** Incubation of INS-1 cells with JI-36 (10^{-6} to 10^{-9} M) for 24–96 h caused a significant and dose-dependent increase in cell proliferation rates. The most effective concentration of the agonist was 10^{-6} M, with a 50% increase after 72 h (Fig. 4A and C). Coincubation of INS-1 cells with the GHRH agonist JI-36 (10^{-6} M) and the GHRH antagonist MIA-602 (10^{-6} M) for 72 h reversed the proliferation-stimulating effect of the agonist.

**Cell Apoptosis Studies on Insulinoma Cells.** Incubation of INS-1 cells with JI-36 (10^{-6} to 10^{-9} M) for 24–96 h resulted in a significant decrease in degree of cell apoptosis as measured by the reduction of activity of caspases 3 and 7. The maximal antiapoptotic effect was seen after 72 h; the most effective concentration of the agonist causing this effect was 10^{-6} M (Fig. 4B and C).

**Determination of Islet Number and Islet Volume.** Cultures of isolated rat islets in the presence of JI-36 showed no relevant change in number of islets over time compared with control islets (Fig. 5A). Calculation of islet equivalents (IEQ) by relative conversion into islets of 150 μm diameter showed a significant increase in IEQ/islet ratio, indicating a relative “islet growth” after 48 h, and up to 72 h, following exposure to JI-36 (Fig. 5B).

Immunohistochemical staining of the islets, after 72 h in culture with JI-36, for insulin and the proliferation marker Ki-67, showed colocalization of the two markers, indicating an induced proliferation, specifically although not exclusively in β-cells (Fig. 5C and D).

**Measurement of Islet Membrane Integrity.** Rat islets were evaluated by fluorescent microscopy using FDA/PI staining. We observed no difference in islet viability between the groups after 24, 48, and 72 h in culture (72-h time point: 93 ± 2.2% for control islets, 96 ± 3.3% for islets exposed to JI-36; n = 4). Morphological appearance following dithizone staining also did not differ between treatment groups.

**Effects of JI-36 on Glucose-Stimulated Insulin Secretion.** In a static model of glucose-stimulated insulin secretion, exposure to JI-36...
glycemic, two had impaired graft function, and one showed graft parison, in the control group, only three animals were normoglycemic at day 25, glucose levels reaching the range of normal healthy mice. When posed to agonist JI-36 consistently performed better, with blood rations tested, animals transplanted with islets previously ex-

for 48 h resulted in a slight increase of insulin release into the culture media after 1 h at basal (3.3 mM) glucose concentration as compared with control (2.3 ± 0.5 ng/mL vs. 1.7 ± 0.1 ng/mL; n = 5). Upon stimulation with high levels of glucose (16.7 mM), insulin release from treated islets was significantly increased (3.6-fold) relative to insulin release at basal glucose concentration, whereas untreated islets augmented insulin release only 1.5-fold (8.2 ± 0.2 ng/mL compared with control 2.6 ± 0.2 ng/mL; n = 5; P < 0.001; Fig. 6). Thus, treatment of rat islets in vitro more than doubled total insulin release upon stimulation. 

Performance of Islets Exposed to JI-36 in Vivo. For all islet preparations tested, animals transplanted with islets previously exposed to agonist JI-36 consistently performed better, with blood glucose levels reaching the range of normal healthy mice. When evaluated at day 25, five of six animals from the JI-36 group were “cured,” and one animal showed partial graft function. In comparison, in the control group, only three animals were normoglycemic, two had impaired graft function, and one showed graft failure (Fig. 7A). In the i.p. glucose tolerance test (IPGTT), the JI-36 group showed an insulin response comparable to that of normal healthy mice, whereas control islets in animals, classified as cured on the basis of attaining normoglycemia before challenge, exhibited delayed and inadequate responses to glucose challenge (Fig. 7B).

Islet grafts retrieved on day 27 after transplantation were immuno-

Discussion

The main finding of the present study is that GHRH agonist JI-36 improves β-cell survival and growth as well as metabolic function. We have shown the expression of mRNA and protein for GHRH in both rodent and human islets. The GHRH agonist reduced programmed cell death of β-cells. This was reversed by an antagonist of GHRH. Finally, pretreatment with GHRH agonist improved β-cell engraftment and metabolic function of islets following transplantation under the kidney capsule in the streptozotocin-induced diabetic mice. Furthermore, islets treated with the GHRH agonist before transplantation into diabetic NOD-SCID mice were able to produce normoglycemia in these mice earlier and more consistently than islets sham-treated without JI-36. In addition, JI-36 exposed islets showed a stronger response upon glucose challenge compared with untreated islets in vitro and in vivo.

GH itself and IGF1, as well as GH-releasing peptides such as ghrelin and other GH secretagogues, have been shown to increase β-cell proliferation in transplanted human and fetal rat islets (25, 26). This study, however, shows the potential role of a GHRH agonist in islet cell proliferation and survival. The detection of the GHRH receptor on β-cells in rat and human islets supports the view that GHRH may exert a direct signal transduction within the pancreas independent and/or in addition to the effects mediated by the GH/IGF1 pathways. Though ghrelin and other GH secretagogues may have pleiotropic actions with potentially unexpected side effects, the administra-

GH releasing hormone, corticotrophin-releasing hormone, on β-cell proliferation (27), further emphasizing an important connection between the hypothalamic-pituitary axis and the integrity of insulin-producing cells in the pancreas. Synthetic agonists of GHRH such as JI-36 are more potent and longer acting than native GHRH or other growth factors. This may open new therapeutic options. Because there are millions of patients with type 1 diabetes, and the availability of pancreatic islet donors is extremely limited, reaching less than a few hundred per year, there is a desperate need for the development of methods for increasing the efficiency of β-cell function and islet cell mass. In vitro expansion of islet cell function and mass by the use of growth factors is therefore of great interest. If future studies can show that this strategy can be safely applied in vivo, treatment with GHRH analog may have a tremendous impact also on the prevention and treatment of type 2 diabetes patients. A major feature of diabetes mellitus type 2 is the progressive loss of β-cell mass over time, very similar to the situation with transplanted human islets.

We and others have previously shown that by improving the quality of islets and by a careful quality control of the islets before transplantation, the results can be substantially improved (2). Furthermore, multiple studies performed recently have clearly shown that β-cells are able to replicate under basal conditions and that β-cell mass can be augmented in response to a variety of physiological and/or pathophysiological stimuli (28). Indeed, it has become obvious that the major source of new β-cells during adult life is more likely due to the proliferation of preexisting β-cells than the differentiation of progenitor or stem cells in the pancreas (29).
Therefore, improving β-cell function and replication in vivo may be an important therapeutic strategy for both the prevention and the cure of diabetes mellitus. Although our study was mainly performed in rodents, we have also shown expression of the receptor in human islet cells. On the basis of previous studies with other growth factors, it is appropriate to extrapolate that human islets will have the same potential to expand and improve islet cell mass in a fashion similar to the results observed in our animal models. In addition to refining quality of islet cells and islet cell function before transplantation, it may be possible to improve islet engraftment and reduce the number of islets needed for a successful outcome by using a short-term in vivo exposure to the agonist. Previous work has shown that temporary systemic administration of growth factors such as hepatocyte growth factor (HGF) may improve graft survival and blood glucose control in vivo (30). This, however, requires further study in vivo to adequately address safety issues and the risk of uncontrolled proliferation and tumorigenesis.

In summary, the current long-term efficacy of clinical islet transplantation is rather low. One of the major underlying factors for this outcome is the loss of islet mass over time. Therefore, the exploitation of mechanisms promoting islet proliferation and growth is critically important for further progress in the field. The application of synthetic GHRH agonist for islet proliferation in vitro as well as graft function and survival in vivo in therapies of diabetes, and our study showing the importance of local autocrine and paracrine GHRH in β-cell regulation and growth, suggest a promising regenerative therapeutic potential for patients with diabetes.

Materials and Methods

Peptide Analogs Preparation. GHRH agonist JI-36 and GHRH antagonist MIA-602 were synthesized in the laboratory of author A.V.S. (17, 19, 20).

Rat Insulinoma Cell Line. Rat insulinoma cells (INS-1) were cultured in RPMI medium 1640 (PAA) supplemented with 2 mM L-glutamine, 10% FBS, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, and 100 U/mL penicillin-streptomycin (Gibco) in a humidified 5% CO2,95% O2 atmosphere at 37 °C. The culture medium was changed every other day. Cells were grown for 72 h before experimentation. GHRH agonist JI-36 (10−8 to 10−6 M) and GHRH antagonist MIA-602 (10−6 to 10−7 M) were used for 24–96 h, respectively.

Isolation of Rat Pancreatic Islets. Pancreatic islets were isolated from male Wistar rats according to guidelines established by the University of Dresden.

Fig. 5. Effect of JI-36 on islet number and islet size in vitro (n = 4). (A) The number of islets decreased slightly over time in culture, with no difference between treatment group and control. The bars represent the percentage of islet number compared with islet yield right after isolation (t0). (B) When converted to islet equivalents (IEQ), a significant difference between JI-36-treated islets and controls was seen after 24 h and continued to increase over time. Gray bars represent control group (n = 4), black bars represent JI-36-treated islets (n = 4). *P < 0.05. (C and D) Immunostaining of islet serial sections for insulin (C, brown staining) and Ki-67 (D, brown staining) showed colocalization (arrowheads) of the proliferation marker within β-cells.

Fig. 6. Effect of GHRH agonist JI-36 on glucose-stimulated insulin secretion. After equilibration at 3.3 mM glucose, islets were stimulated with high glucose concentration of 16.7 mM for 1 h. Exposure to JI-36 did not cause a relevant difference in insulin secretion at basal conditions. Glucose challenge resulted in significantly increased insulin release 3.6-fold relative to insulin release at basal glucose concentration when compared with untreated islets that increased insulin release 1.5-fold relative to insulin release at basal glucose concentration (n = 5). Overall, pretreatment with JI-36 resulted in a more than double insulin release upon glucose stimulation compared with control (***P < 0.001).
creatic duct. Islets were separated from exocrine tissue by centrifugation on a continuous-density Biocoll gradient (Biochrom) in a COBE 2991 cell processor. For determination of purity and islet yield, islet samples were stained with dithizone (Sigma-Aldrich) and sized using an eyepiece reticle and inverted microscope. Islets were cultured in CMRL 1066 (Mediatech) containing 2.5% human serum albumin at 37 °C in a 5% CO2 incubator before experimentation.

Islet Equivalent Determination. Triplicate samples of 100–300 islets were stained with dithizone (Sigma-Aldrich), which binds zinc ions present specifically in islet β-cells, and sized using an eyepiece reticle and inverted microscope (32). All islets with a diameter <50 μm were divided into classes of 50-μm increments (i.e., 50–100, 100–150, 150–200, etc.) for calculation of islet equivalents (IEQ). Each diameter class was converted into the mean volume of 150-μm diameter islets by a relative conversion factor. These factors allow converting the total islet number from any preparation into IEQ.

Exposure of INS-1 Cells and Rat and Human Islets to GHRH Analogues. INS-1 cells were grown for 72 h before experimentation; islets were collected immediately after the isolation procedure and divided into three treatment groups: (i) culture media with vehicle (DMSO) as a control group, (ii) culture media containing GHRH agonist JI-36 (10−6 M), and (iii) culture media with JI-36 plus GHRH antagonist MIA-602 (10−6 M). Media change and addition of the analogs was performed after 24 h and 48 h in islet cultures and every other day in INS-1 cell cultures.

Fluorescein Diacetate-Propidium Iodide Viability Staining. Small aliquots of islets were transferred in PBS-containing Petri dishes. Fluorescein diacetate (FDA) and propidium iodide (PI) were added to the samples at a final concentration of 0.5 and 75 μM, respectively. Using a fluorescence microscope, 100 islets were assessed for cell viability by estimating the percentage of viable cells (green) vs. nonviable cells (red) within each islet. The percentage of viable cells was then calculated (33).

Measurement of Insulin Secretion by Static Challenge with Glucose. For static insulin secretion in response to glucose challenge, islets were transferred into Petri dishes containing oxygenated Krebs–Ringer bicarbonate buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7 H2O, 2.5 mM CaCl2·2 H2O, 25 mM NaHCO3, 0.25% BSA) and preincubated in 3.3 mM glucose at 37 °C (5% CO2) for 30 min. Groups of 8–10 islets from the equilibration cultures were transferred to fresh oxygen-saturated media containing either 3.3 or 16.7 mM glucose and then incubated an additional 60 min in a 37 °C water bath with gentle shaking. Secreted insulin in the media was measured by ELSA (Millipore) and values normalized to extracted islet DNA (Quant-iT Picogreen; Invitrogen).

In Vivo Islet Functional Assessment. NOD-SCID mice (MTZ breed) with induced diabetes were used as islet recipients following guidelines established by the University of Dresden Institutional Animal Care and Use Committee. Diabetes was induced by a single i.p. injection of 180 mg/kg streptozotocin (Sigma-Aldrich). Serum glucose was then measured daily using an Ascensia Elite glucometer (Bayer). Mice were considered diabetic if nonfasting blood glucose was >350 mg/dL for 2 or more consecutive d. Rat islet preparations were used for transplantation. Islets from each preparation were divided into two groups, and JI-36 (10−6 M) or vehicle (DMSO) was added to the culture media. Islets were cultured for 48 h before transplantation. After culture, samples of 300 IEQ were washed in transplant media (Ringer acetate with 5% glucose and 10% FBS) and transplanted to beneath the left kidney capsule. The animal experiments and housing were in accordance with institutional guidelines and German animal regulations.

Posttransplant Follow-Up. The mice were observed for 30 d after transplantation. The nonfasting blood glucose levels were measured daily during the first week and twice a week thereafter. On day 25, mice were subjected to an IPGTT. Two days later, grafts were removed. This led to a recurrence of the hyperglycemia to the result of islet graft function.

Institutional Animal Care and Use Committee. Animals were anesthetized by 3% isoflurane; digestion solution (Collagenase V; Sigma-Aldrich) was injected in situ via the pancreatic common bile duct. Islets were purified by centrifugation on a discontinuous Ficoll gradient (Mediatech). Purified islets were maintained in culture media (CMRL 1066; Mediatech) supplemented with 10% FBS at 37 °C in a 5% CO2 incubator. Volume and purity were determined by microscopic sizing after staining with dithizone (Sigma-Aldrich).

Isolation of Human Pancreatic Islets. Human pancreata from cadaver donors were obtained through Eurotransplant following consent for research use obtained from the next of kin and authorization by the German Foundation for Organ Transplantation. Islets were isolated using a modification of the automated Ricordi method (31). Briefly, collagenase NB1, neutral protease (Serva Electrophoresis), and DNase (Roche) were infused into the main pan-
electron microscopy, measurement of cell proliferation, caspase activity, and statistical analysis, see SI Text.

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Total RNA was isolated from frozen INS-1 cells or islets of test. Differences were considered significant. Cell lysis reagent (Sigma-Aldrich) containing 0.05% Triton X-100 rat or human cell lysis buffer (Invitrogen), 0.25 mM of each deoxynucleotide triphosphate (Promega), 1 unit of Platinum Taq DNA Polymerase (Invitrogen), 1 mM of each primer (Promega), 1 μL solution containing 1.5 mM MgCl₂, 1× PCR buffer (Invitrogen), and 0.5 μM of each of the different primers. Samples were heated for 5 min at 94 °C, then subjected to 15 s at 94 °C, 15 s at 65 °C, and 15 s at 72 °C for 40 cycles and finally amplified at 72 °C for 1 min. Following agarose gel electrophoresis and ethidium bromide staining, bands were visualized under UV light.

Primers sequences (5′–3′) used were rat (sense: 5′-ccaaaccggctctgggtg-3′; antisense: 5′-ggctcagcaatcaggg-3′) and human (5′-ttgctgacagctgctactg-3′; 5′-ggctcagcaatcaggg-3′) GHRH-R primers.

Western Blot Analysis. Western blot analysis was performed as previously described (1). Briefly, INS-1 cells and rat and human islets were washed twice in ice-cold PBS and lysed in ice-cold CellLytic™ M Cell Lysis Reagent (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich), 0.5 μM of each of the different primers. Samples were heated for 5 min at 94 °C, then subjected to 15 s at 94 °C, 15 s at 65 °C, and 15 s at 72 °C for 40 cycles and finally amplified at 72 °C for 1 min. Following agarose gel electrophoresis and ethidium bromide staining, bands were visualized under UV light.

Western blot analysis was performed as previously described (1). Briefly, INS-1 cells and rat and human islets were washed twice in ice-cold PBS and lysed in ice-cold CellLytic™ M Cell Lysis Reagent (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). Cell lysates were matched for protein content; equal amounts of protein were loaded on each lane, separated by SDS/PAGE and transferred to nitrocellulose membrane. After blocking, membranes were immunostained with rabbit polyclonal GHRH antibodies (ab-28692 against human and ab-76263 against mouse and rat; Abcam). Bound primary antibody was detected using a Western Blot Detection Kit (Invitrogen) with the appropriate secondary antibody. Chemiluminescence signals were read with the GeneGnome Chemiluminescence detector (Syngene).

Immunohistochemical Analysis. Samples of 50–100 rat or human islets were fixed in 4% paraformaldehyde for 1 h and processed in paraffin. Serial sections of 4 μm were stained using an automated immunostainer (BenchMark; Ventana) according to the manufacturer’s protocols. Primary antibodies were rabbit polyclonal GHRH antibodies (ab-28692 against human and ab-76263 against mouse and rat; Abcam), mouse monoclonal antibody to insulin (Clone AE9D6, unconjugated from BioGenex Laboratories), and monoclonal mouse anti-human Ki-67 antigen (Clone MIB-1, unconjugated from DakoCytomation). The signal was amplified using the Ventana amplification kit and visualized using avidin-biotin labeling and 3,3′-diaminobenzidine. Slides were counterstained with hematoxylin. Staining with isotype control antibodies was performed to confirm staining specificity.

Electron Microscopy. INS-1 cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and refixed in 1% osmium tetroxide solution as described previously (2). After dehydration in an ascending ethanol series, specimens were embedded in Epon Ultrathin sections (60 nm) were prepared. To obtain a suitable contrast, slices were stained with lead acetate and uranyl acetate and analyzed using an electron microscope (Zeiss 906).

Measurement of Cell Proliferation. Cell proliferation was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) kit according to the manufacturer’s description.

Caspase Activity. Activity of caspases 3 and 7 was assessed using Caspase-Glo 3/7 Assay (Promega) following the manufacturer’s description.

Statistical Analysis. In all experiments, statistical differences between experimental groups vs. appropriate controls were determined using ANOVA. Data are presented as mean ± SEM. Statistical significance was tested by ANOVA with Bonferroni’s or Student’s t test. Differences were considered significant at values of P < 0.05.
