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Glucagon-Like Peptide 1 Inhibits Cell Apoptosis and Improves Glucose Responsiveness of Freshly Isolated Human Islets

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The peptide hormone, glucagon-like peptide 1 (GLP-1), has been shown to increase glucose-dependent insulin secretion, enhance insulin gene transcription, expand islet cell mass, and inhibit β-cell apoptosis in animal models of diabetes. The aim of the present study was to evaluate whether GLP-1 could improve function and inhibit apoptosis in freshly isolated human islets. Human islets were cultured for 5 d in the presence, or absence, of GLP-1 (10 nM, added every 12 h) and studied for viability and expression of proapoptotic (caspase-3) and antiapoptotic factors (bcl-2) as well as glucose-dependent insulin production. We observed better-preserved three-dimensional islet morphology in the GLP-1-treated islets, compared with controls. Nuclear condensation, a feature of cell apoptosis, was inhibited by GLP-1. The reduction in the number of apoptotic cells in GLP-1-treated islets was particularly evident at d 3 (6.1% apoptotic nuclei in treated cultures *vs.* 15.5% in controls; $P < 0.01$) and at d 5 (8.9 *vs.* 18.9%; $P < 0.01$). The antiapoptotic effect of GLP-1 was associated with the down-regulation of active caspase-3 ($P < 0.001$) and the up-regulation of bcl-2 ($P < 0.01$). The effect of GLP-1 on the intracellular levels of bcl-2 and caspase-3 was observed at the mRNA and protein levels. Intracellular insulin content was markedly enhanced in islets cultured with GLP-1 *vs.* control ($P < 0.001$, at d 5), and there was a parallel GLP-1-dependent potentiation of glucose-dependent insulin secretion ($P < 0.01$ at d 3; $P < 0.05$ at d 5). Our findings provide evidence that GLP-1 added to freshly isolated human islets preserves morphology and function and inhibits cell apoptosis. (*Endocrinology* 144: 5149–5158, 2003)

SEVERAL BIOLOGICAL FEATURES of glucagon-like peptide 1 (GLP-1) have led to propose this peptide hormone as an ideal candidate for the treatment of diabetes (1). GLP-1 lowers postprandial hyperglycemia via three independent mechanisms: increases insulin secretion, inhibits glucagon release, and inhibits gastrointestinal motility. Perhaps even more important is the observation that the insulin secretory action of GLP-1 is regulated by the plasma concentration of glucose, virtually preventing the possibility of developing reactive hypoglycemia while inducing the release of insulin (2). Finally, it is of significant clinical relevance the observation that GLP-1 retains its glucose-lowering activity in patients with diabetes, even many years after clinical onset of the disease, when islet β-cells are no longer responsive to other pharmacological insulin-secreting agents (3).

In addition to these very well-characterized properties, novel biological actions of GLP-1 have recently been proposed. It has been demonstrated that the role of GLP-1 extends beyond its glucose-lowering action in response to food ingestion and included the *de novo* synthesis of proinsulin (2). These findings substantially enrich, and somehow reframe, the general understanding of the role of GLP-1 in the physiology of islet cells. Thus, the GLP-1-dependent regulation of glucose homeostasis appears to be based on biological mechanisms far more complex than the simple modulation of insulin secretion. Indeed, GLP-1 also affects the expression of insulin and other β-cell-specific genes whose products are involved in the regulation of glucose utilization (4, 5). The mechanism by which GLP-1 modulates the β-cell-specific gene expression has only in part been elucidated, and it is known to require the activation of the homeodomain transcription factor IDX-1 (6).

Findings from *in vivo* studies have shown that the beneficial long-lasting effects of GLP-1 can be also partly attributed to changes in β-cell mass (4). In Wistar rats the age-dependent decline in β-cell function and the consequent impairment of glucose tolerance were reversed by constant sc infusion of GLP-1 (5). Similarly, Xu *et al.* (7) demonstrated that an analog of GLP-1, termed exendin-4, was able to increase islet mass in adult animals previously subjected to subtotal pancreatectomy.

Recent reports have also shown that GLP-1 has antiapoptotic properties in addition to its effect on the expression of islet-specific genes and islet cell mass. In diabetic ZDF rats as well as in streptozotocin-induced diabetic mice, treatment with GLP-1 or exendin-4 protects β-cells from apoptosis (8, 9). *In vitro* studies using insulin-secreting cells (10) or fibroblast (9) showed that the protective action of GLP-1 was not

Abbreviations: DAPI, 6-Diamino-2-phenylindole; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GLP-1, glucagon-like peptide 1; 3-D, three-dimensional.

an indirect effect obtained by normalizing the ambient glucose levels or promoting the secretion of insulin, but it was rather a direct effect on cell viability, and it was mediated by an increase expression of antiapoptotic proteins and a downregulation of proapoptotic proteins.

The studies of the antiapoptotic action of GLP-1 conducted to date have used animal models or cell lines; no studies have investigated yet the antiapoptotic effect of GLP-1 on primary cultures of human islets. The aim of the present study was to investigate the capability of GLP-1 to preserve viability and function of freshly isolated human islets.

Materials and Methods

Tissue procurement and islet isolation

The experimental protocol was carried out using islets unsuitable for transplantation due to serologic testing or inadequate islet yield. Islets collected from three independent donors were used for the study. Islet isolation was performed by the Islet Core Laboratory of the Diabetes Branch at the National Institutes of Health using the method of Ricordi and co-workers (11). After purification on a Ficoll gradient, the top interface containing the largest quantity of islets (>80%) was harvested for experimental procedures hereafter described. The purity of the islets was assessed by staining with dithizone (Sigma, St. Louis, MO), whereas viability was determined by trypan blue exclusion test. We used only islet preparations with a greater than 80% of viability and 90% of purity for the present study.

Human pancreatic islet in vitro culture

Purified human islets were divided into three groups and cultured, respectively, for 1, 3, and 5 d using M199 medium (Life Technologies, Inc., Rockville, MD) in the presence of 6 mM glucose, 10% fetal calf serum (FCS) (Life Technologies, Inc.), 0.1 mM diprotin-A (Bachem/Peninsula, San Carlos, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 C with 5% CO_2. Each group was subdivided into two categories: one category was treated with 10 nM GLP-1 (Bachem, King of Prussia, PA), added every 12 h in the presence of 0.1 mM diprotin-A, and the second category was cultured in GLP-1-free medium. At the end of d 1, 3, and 5, a glucose-induced insulin secretion test was performed by changing the culture medium to a 15-mM glucose-containing medium. A sample of the culture medium as well as the cell pellet was kept at different time points: 0, 10, 20, and 60 min to perform insulin quantification by RIA (Linco Research Inc., St. Charles, MO). Islet proteins were extracted using M-PER mammalian protein extraction buffer (Pierce, Rockford, IL) in the presence of the Halt protease inhibitor cocktail (Pierce) and stored at −80 C. The extracted proteins were quantified with the protein quantification kit (Dojingo, Gaithersburg, MD). Under the same culture conditions as described above, a sample of human islets were plated into Lab-Tek II Chamber slides (VWR Scientific Product, Willard, OH) for 1, 3, and 5 d and then fixed in 4% paraformaldehyde (Sigma) to perform immunofluorescence studies.

Morphology study

Approximately 200 islets per culture condition were plated and cultured for 1, 3, or 5 d in the presence of GLP-1 or vehicle. Their morphology was studied by light microscope photography using a CK-2 microscope (Olympus, Melville, NY). Pictures were taken with an camera (model C-35AD-4, Olympus, Tokyo, Japan) at the time of plating and harvesting.

Detection of bcl-2 and capsase-3 mRNA levels by RT-PCR

After culturing human islets for 1, 3, and 5 d, the cellular pellets were isolated to perform direct extraction of mRNA following the manufacturer protocol for the Oligotex Direct mRNA extraction (QIAGEN Inc., Valencia, CA). One hundred nanograms of mRNA from each islet sample were used for cDNA first-strand synthesis performed according to the Prostar first-strand RT-PCR kit (Stratagene, La Jolla, CA). The cDNA was then used for the amplification of caspase-3, bcl-2, and β-actin. Before selecting the number of PCR cycles for the amplification of each transcript, we performed a PCR titration subjecting the cDNA to 20, 25, 30, 35, and 40 rounds of amplification.

For caspase-3 RT-PCR, we designed a set of primers able to amplify a fragment of 315 bp, which contain the sequence used for the production of a monoclonal antibody specific for the active form of caspase-3: sense 5′-tttttcagaggggatcgttg-3′ and antisense 3′-aattctgttgccacctttcg-5′. The reaction mixtures were first denatured at 95 C for 1 min and then subjected to PCR amplification using the following conditions: 95 C for 30 sec and 54 C (annealing/extension) for 1.5 min. Samples were incubated for an additional 10-min extension at 70 C. For bcl-2 RT-PCR, we used the following primers: sense 5′-ctgcgaagaaccttgtgtga-3′ and antisense 3′-tgtccctaccaaccagaagg-5′. The size of the bcl-2 PCR product was 215 bp in length. The reaction mixtures were first denatured at 95 C for 1 min. Different from the amplification for caspase-3, the PCR for bcl-2 was performed using an annealing temperature of 60 C for 1.5 min; all other conditions were kept the same. The size of the PCR products was compared with a low DNA Mass ladder (Amersham Biosciences, Piscataway, NJ). The identity of amplified PCR products was confirmed by DNA sequencing (Biotech Core, Inc., San Francisco, CA).

RT-PCR for β-actin was used as a control, and the relative abundance of bcl-2 and caspase-3 mRNA was obtained by normalizing the intensity of the amplified band for the level of β-actin for each culture condition. The following primers and PCR conditions were used: sense 5′-gtggggcgccccaggcacca-3′ and antisense 5′-ctccttaatgtcacgcacgatttc-3′. The reaction mixtures were first denatured at 95 C for 1 min and amplified at the following conditions: 95 C for 30 sec and 58 C (annealing/extension) for 1.5 min. Samples were incubated for an additional 10-min extension at 70 C. The human β-actin final PCR product size was 340 bp.

Immunofluorescence studies

The tissue slides were pretreated with 3% H_2O_2 (Sigma) in 100% methanol (J.T. Backer; Phillipsburg, NJ) for 10 min, washed three times in PBS (Sigma), treated with 0.1% Triton (Sigma) for 10 min, and washed again in PBS three more times. Blocking solution was added for 1 h. All of the above procedures were conducted at room temperature. The excess blocking solution was removed, and the primary antibodies were added and incubated overnight at 4 C. The following day the slides were washed in PBS, and the secondary antibodies were added. All of the antibodies were diluted in PBS. The nuclear staining was performed using mounting medium with 4′, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA). The staining of islets in the absence of the primary antibody was used a control for all immunofluorescence studies.

The results were analyzed using a light and fluorescence AX-70 microscope (Olympus) with a fluorescein isothiocyanates (FITC)-conjugated standard filter set (520 ± 20 nm) and a rhodamine standard filter set (>620 nm). The images were captured with an Apogee digital camera model KX-2E (Scientific Instruments Co., Culver City, CA) and processed with an Image-Pro focus control computer program PC (Scientific Instruments).

Double staining for Bcl-2 and active form of caspase-3. Detection of bcl-2 and the active form of caspase-3 was performed according to the protocol described above. We used 10% normal goat serum (Chemicon International, Inc., Temecula, CA) as blocking solutions and the following primary antibodies: purified rabbit antiactive caspase-3 monoclonal antibody (BD PharMingen, Los Angeles, CA) diluted 1:200 and the purified anti-bcl-2 monoclonal antibody (BD PharMingen) diluted 1:200. The antibody against caspase-3 recognizes the 18-kDa subunit of the human active caspase-3 and does not recognize the 32-kDa procaspase (12). The secondary antibodies were rat antimouse IgG1 FITC-conjugated (Zymed, San Francisco, CA) diluted 1:50 and goat antirabbit rhodamine-conjugated antibody (Chemicon) diluted 1:100.

Although the direct detection, by Western blot analysis or RIA, of bcl-2 and caspase-3 levels are the gold standard for measuring the intracellular protein content, the scarcity of tissue available for this study directed us to identify an alternative approach to obtain an indirect quantitative measure of bcl-2 and caspase-3 levels in the various culture conditions. In this study, the quantification of antiapoptotic and proapoptotic cellular proteins was extrapolated by analyzing the variance

of the signal released by the fluorochrome molecules FITC and rhodamine (for bcl-2 and caspase-3, respectively), as determined from at least five independent immunostainings of islets from independent donors. The numerical values of color density were obtained using the software ImageJ (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/nih-image/).

Immunostaining for insulin. For insulin immunostaining, the blocking solution was prepared using 10% of normal goat serum. The first antibody, a guinea pig antiinsulin polyclonal antibody (Dako Corp., Carpinteria, CA), diluted 1:80, was added and incubated overnight at 4 C. The secondary antibody, a goat anti-guinea pig rhodamine-conjugated antibody (Chemicon) diluted 1:100, was incubated for 2 h at room temperature.

DAPI staining. Islets were fixed as described above and rinsed in PBS before the addition of the mounting medium containing the DAPI staining (Vector Laboratories). The slides were then examined using an Apogee digital camera (model KX2E) attached to the Olympus BH-2 microscope and processed with an Image-Pro computer program (Media Cybernetics, Inc., Silver Spring, MD).

Point counting of for double-positive cells (DAPI and insulin-positive cells) was used for a quantitative analysis of the number of islet cells containing insulin at various time points. By dividing the number of double-positive cells (DAPI+/insulin+) cells by the number of total DAPI+ cells in each islet, we determined the number of living cells that contained insulin at the various time points and under the different culture conditions (with or with GLP-1).

Insulin synthesis and secretion

A glucose-induced insulin secretion test was performed on each group of islets at the end of d 1, 3, and 5. An aliquot of the culture medium as well as the cell pellet was collected at different time points (0, 10, 20, 60 min) after glucose stimulation and used to detect the intracellular content of insulin by RIA assay (Linco). The insulin values were normalized for the total protein content obtained from the cell pellet extracted with M-PER mammalian protein extraction buffer (Pierce) in the presence of the Halt protease inhibitor cocktail (Pierce). Statistical analysis was expressed as mean ± SE obtained comparing the two groups of islets (control and GLP-1), at d 3, stimulated with 15 mM glucose at 0, 10, 20, and 60 min.

Statistical analysis

The data were expressed as mean ± SE. Significance of the data was evaluated by one-way ANOVA or Student's *t* test, as specifically indicated in the figure legends. Comparison between GLP-1-treated and control cultures over time was performed using Bonferroni-Dunn *post hoc* analysis.

Results

Morphology studies

Human pancreatic islets showed important morphological changes during the 5 d of culture. Islets cultured for only 1 d maintained their physiological spherical shape. As the number of days in culture increased, many islets showed a progressive loss of their typical macroscopic structure; this was associated with the disappearance of the acellular membrane that surrounds them. At d 5 many cell aggregates were no longer organized in the three-dimensional (3-D) structure typical of islet cells aggregates, and many of them showed the two-dimensional structure typical of a cell monolayer. These observations were in contrast with those derived from the islets treated with GLP-1, which were able to maintain the 3-D organization for a longer period of time (Fig. 1). Although the number of cell aggregates did not vary signifi-

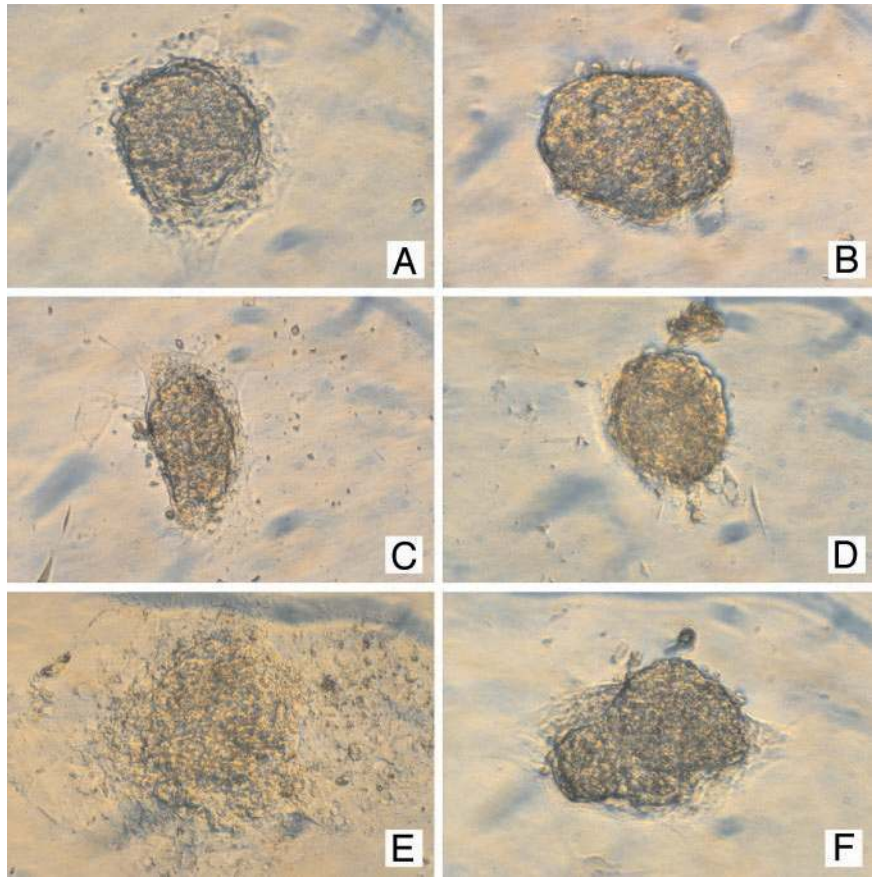

FIG. 1. Islets cell morphology. Human islets were cultured for 1, 3, and 5 d in M199 medium, with 6 mM glucose, 10% FCS, and 0.1 mM diprotin-A and in the presence, or absence, of GLP-1 (10 nM, added every 12 h). Human islets on d 1, control (A) and GLP-1-treated islets (B); d 3, control (C) and GLP-1 treated (D); and d 5, control (E) and GLP-1 treated (F). Pictures are representative of islets morphology as observed by culturing human islets from three independent donors. Magnification, ×20.

cantly among the different treatment groups over the 5 d of culture, we observed that by d 5 in control cultures, there was a reduction of approximately 45% of the number of islets with a preserved 3-D structure, compared with a reduction of approximately 15% in GLP-1-treated islets ($P < 0.01$) (Fig. 2).

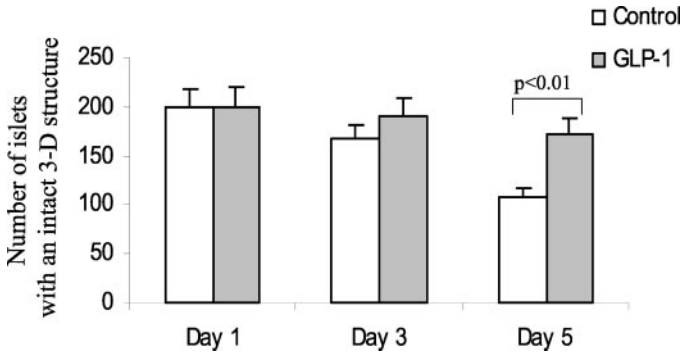

FIG. 2. Number of islets with an intact 3-D structure. Approximately 200 human islets were plated in M199 medium, with 6 mM glucose, 10% FCS, and 0.1 mM diprotin-A and cultured in the presence, or absence, of GLP-1 (10 nM, added every 12 h). The graph shows the number of islets with a preserved 3-D structure in each culture condition by d 1, 3, or 5. Pictures are representative of the morphology of islets form three independent donors. Two independent sets of islets per each culture condition were analyzed by a single observer, using magnification, ×4.

DAPI staining

DAPI staining of GLP-1-treated and control cell cultures showed some important treatment-dependent changes in the morphology of cell nuclei during the 5-d study. Although on d 1 the nuclei of cells from all different culture conditions were homogeneous and regular (Fig. 3), by d 3 the control dishes showed the presence of many cells with condensed nuclei, a characteristic morphological change in cells that is associated with apoptosis. An even greater number of apoptotic cells was observed on d 5. As suggested by the light microscopy observation described above, the addition of GLP-1 to the culture medium had a significant effect on cell viability, with an inhibition of the nuclear morphological changes that are characteristic of apoptotic cell death. The protective effect of GLP-1 was readily detectable on d 3 and persisted on d 5.

Bcl-2/active caspase-3 immunofluorescence

Bcl-2 is an antiapoptotic molecule capable of inhibiting the proapoptotic actions of the caspases. The activation of caspase-3 is associated with the final steps in the sequence of events that leads to cell apoptosis. The relative abundance of bcl-2 and the active form of caspase-3 could therefore be used to determine whether the balance between antiapoptotic and proapoptotic agents favors cell survival or cell death. The double immunofluorescence for bcl-2/caspase-3

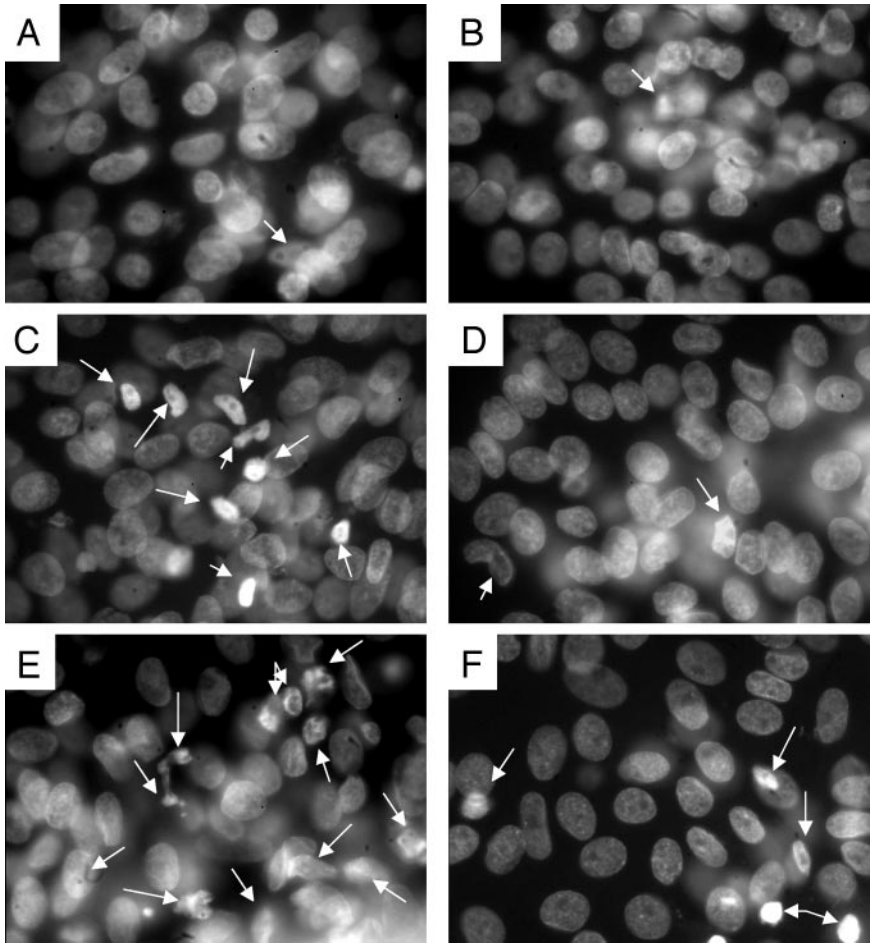

FIG. 3. DAPI staining of islet cells nuclei. Human islets were cultured for 1 (A and B), 3 (C and D), and 5 (E and F) d in M199 medium, with 6 mM glucose, 10% FCS, and 0.1 mM diprotin-A and in the presence (B, D, and F) or absence (A, C, and E) of GLP-1 (10 nM, added every 12 h). Pictures (×100) are representative of cell apoptosis, as observed by culturing human islets from three independent donors. The *arrows* show the condensed nuclei of apoptotic cells.

showed that GLP-1 strongly induces the expression of bcl-2 and reduces the expression of caspase-3, underlying the protective role of GLP-1 against cell apoptosis. The effect of GLP-1 was detectable from d 1 of culturing (Fig. 4, d 1, E–H), more evident on d 3 (Fig. 4, d 3, E–H), and still present on d 5 (Fig. 4, d 5, E–H). In contrast, control islets, when compared with GLP-1 treated, showed a lower expression of bcl-2 on d 1 (Fig. 4, d 1, A–D), with a decrement on d 3 (Fig. 4, d 3, A–D), and this was even greater on d 5 (Fig. 4, d 5, A–D). This decline in bcl-2 expression was associated with increasing levels of the active caspase-3 expression, and this was particularly evident on d 5.

Quantification analysis of GLP-1 effect on cell apoptosis

DAPI staining was used to quantify cells apoptosis. Five random fields per each slide (containing multiple islets) were analyzed (n = 9 slides); islets from three different donors were studied (n = 3 slides per islets donor). The percentage of cells with condensed nuclei was counted and normalized for the number of nucleated cells in each field studied. Although, independent from the treatment employed, time appeared to be the major determinant for the appearance of apoptotic cells in cultures; GLP-1 had a significant antiapoptotic effect on the viability of islet cells (Fig. 5A). Indeed, on d 3, approximately 15.5% of the cells in control cultures were apoptotic, compared with 6.1% in GLP-1-treated cultures ($P < 0.01$). On d 5, the number of apoptotic cells was 18.9% and 8.9%, respectively, in control and GLP-1-treated islets ($P < 0.01$).

Because the degenerative changes of cell nuclei that are identified by DAPI staining are very late morphological features in the sequence of events that leads to cell death, we also measured the intracellular levels of bcl-2 and capsase-3, which may better reflect the acute antiapoptotic effect of GLP-1. The quantification of bcl-2 and active caspase-3 levels was obtained by a computer-generated analysis of the variance of the signal released by the fluorochrome molecules FITC and rhodamine, respectively, used for bcl-2 and caspase-3 detection. This showed a progressive increase of bcl-2 expression over time in islets treated with GLP-1 (Fig. 5B). In contrast, control islets were characterized by a time-dependent increase in expression of the active form of caspase-3 (Fig. 5C). Furthermore, in GLP-1-treated islets, the expression of the active form of caspase-3 decreased significantly on d 3 and 5, whereas bcl-2 showed a symmetrical increase. These data suggest that both the increase of bcl-2 and the decrease of caspase-3 expression mediate the antiapoptotic action of GLP-1.

Detection of bcl-2 and capsase-3 mRNA levels by RT-PCR

RT-PCR for β-actin, bcl-2, and caspase-3 lead to the amplification of DNA fragments of 340, 315, and 215 bp, respectively (Fig. 6A). Although in control cultures time af-

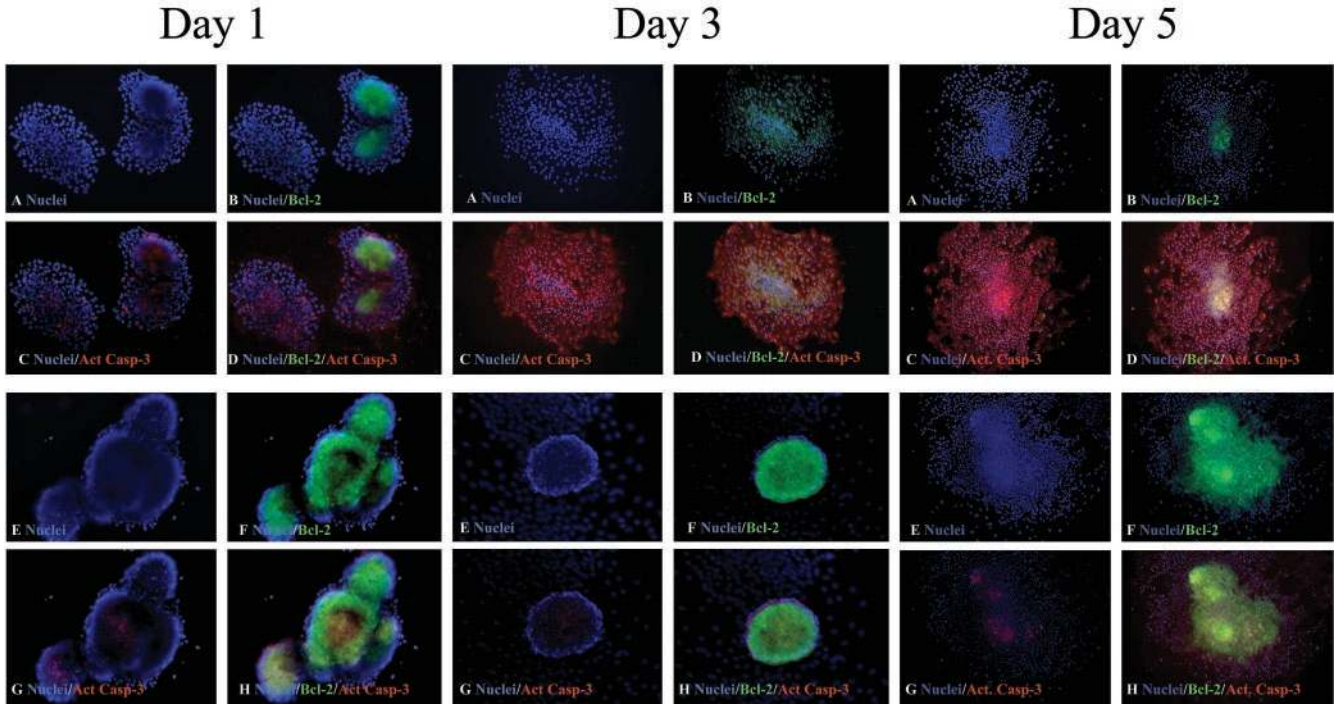

FIG. 4. Expression of bcl-2 and the active form of caspase-3: d 1, 3, and 5. Human islets were cultured in M199 medium, with 6 mM glucose, 10% FCS, and 0.1 mM diprotin-A and in the presence, or absence, of GLP-1 (10 nM, added every 12 h). Immunofluorescence study in control islets (×40). A, DAPI nuclei staining. B, Overlapping of the nuclei staining and bcl-2 (*green*). C, Overlapping of the nuclei staining and caspase-3, active form (*red*). D, Merge of the nuclei, bcl-2, and caspase-3 signals. E–H, Immunofluorescence study in GLP-1-treated islets (×40). E, DAPI nuclei staining. F, Overlapping of the nuclei staining and bcl-2 (*green*). G, Overlapping of the nuclei staining and caspase-3, active form (*red*). H, Merge of the nuclei, bcl-2, and caspase-3 signals. Pictures are representative of bcl-2 and caspase-3 levels, as observed by culturing human islets from three independent donors. Specificity of immunostaining was validated by the lack of fluorescent signal obtained by tissue staining in the absence of the primary antibody.

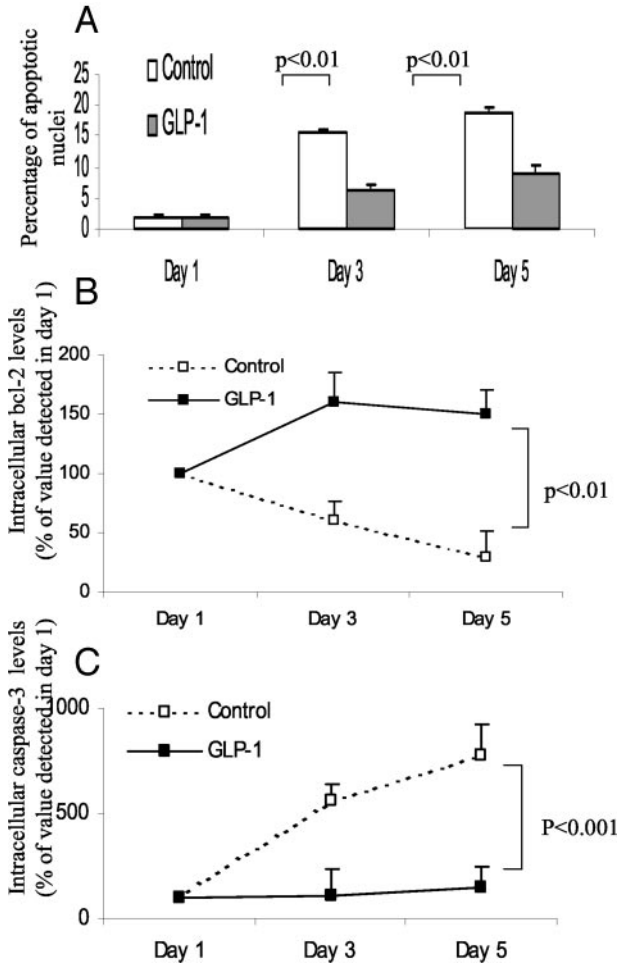

FIG. 5. Quantification of GLP-1 effect on islet cell apoptosis. A, Quantification of condensed nuclei detected with DAPI staining on d 1, 3, and 5. Five random fields per each slide (containing multiple islets) were analyzed (n = 9 slides); islets from three different donors were studied (n = 3 slides per islets donor). The percentage of cells with condensed nuclei was counted and normalized for the number of nucleated cells in each field studied. B, bcl-2 expression in control and GLP-1-treated islets. C, Caspase-3 active form expression in control and GLP-1-treated islets. For B and C, the quantification of antiapoptotic and proapoptotic cellular proteins was extrapolated by analyzing the variance of the signal released by the fluorochrome molecules FITC (bcl-2) and rhodamine (caspase-3), as determined from at least five independent immunostainings of islets. Statistical significance of the data were evaluated by Student's *t* test (A) and ANOVA (B and C).

fected the cellular abundance of the antiapoptotic protein bcl-2, the presence of GLP-1 in the culture medium appeared to maintain the level of bcl-2 mRNA throughout the duration of the study (Fig. 6B, *top panel*). An apposite effect was observed on the mRNA levels for caspase-3, with GLP-1 preventing the time-dependent increase of its intracellular levels (Fig. 6B, *lower panel*). Quantification of the bcl-2 mRNA level (Fig. 6C) and caspase-3 mRNA (Fig. 6D) has been performed.

The bcl-2 and caspase-3 PCR fragments have been analyzed by sequencing, using the same primers previously used for PCR amplification. The results were compared with a gene bank (http://www.ncbi.nlm.nih.gov/BLAST) and showed 100% homology of the RT-PCR products for both Bcl-2 and caspase-3 with their respective gene sequences.

Insulin immunofluorescence

In control islets, there was a progressive reduction of the number of insulin-containing cells. This was evident since d 1 (Fig. 7, d 1, A and B) and became more prominent on d 3 (Fig. 7, d 3, A and B) and d 5 (Fig. 7, d 5, A and B). This observation was in contrast with what was observed in GLP-1-treated cultures, which exhibited a stronger immunofluorescence signal for insulin, and in which the large majority of DAPI-positive cells contained insulin in their cytoplasm. The greater number of insulin-containing cells in GLP-1-treated islets, compared with controls, was observed on d 1 (Fig. 7, d 1, C and D), d 3 (Fig. 7, d 3, C and D), and was still present on d 5 (Fig. 7, d 5, C and D).

By point counting of double-positive cells (DAPI+/insulin+) *vs.* the total number of islet cells (DAPI+ cells), we observed that with time there was a progressive decline in the number of insulin-containing cells per each individual islet examined. However, although by d 5 there were 33 ± 5.2 islet cells that stained for insulin, the presence of GLP-1 in the culture medium increased the number of insulin-containing cells to 68 ± 4.08 ($P < 0.001$) (Fig. 7).

Insulin synthesis and secretion

Exposure of human islets to GLP-1 enhanced their capability of responding to glucose stimulation by secreting insulin. The most significant glucose-dependent secretion of insulin occurred after 3 d of culture, stimulating the islets with 15 mM glucose (Fig. 8). On d 5, all culture conditions showed a decrease in the glucose-dependent secretion of insulin. However, this was less prominent in cultures exposed to GLP-1. Control islets were less sensitive to glucose and showed a delayed (30′-60′) insulin-secretory response (Fig. 8). The data are consistent with those obtained by assaying the cell pellet of the various culture conditions for insulin. This showed that GLP-1 was capable of promoting an increase in the intracellular insulin content (Fig. 9). This observation further supports the evidence that GLP-1 promotes the *de novo* synthesis of insulin and is not simply a glucose-dependent secretion of insulin.

Discussion

The search for a viable β-cell growth factor in the time when pancreatic islet transplantation has become a reality is more imperative than ever. The regulation of islet cell mass involves the integrated functions of β-cell proliferation and neogenesis, with β-cell destruction or apoptosis. Factors that can augment β-cell mass are of particular interest in the field of islet cell transplantation because there are insufficient quantities of islets available for the number of putative recipient candidates (13, 14). Many growth factors are being investigated as potential agents to increase or preserve islet β-cell mass before and after transplantation (15–22). A proproliferative action of GLP-1 on insulin-secreting cells has been shown in both *in vitro* and *in vivo* models (7, 23–34). Recently data from our and other laboratories (8–10) have

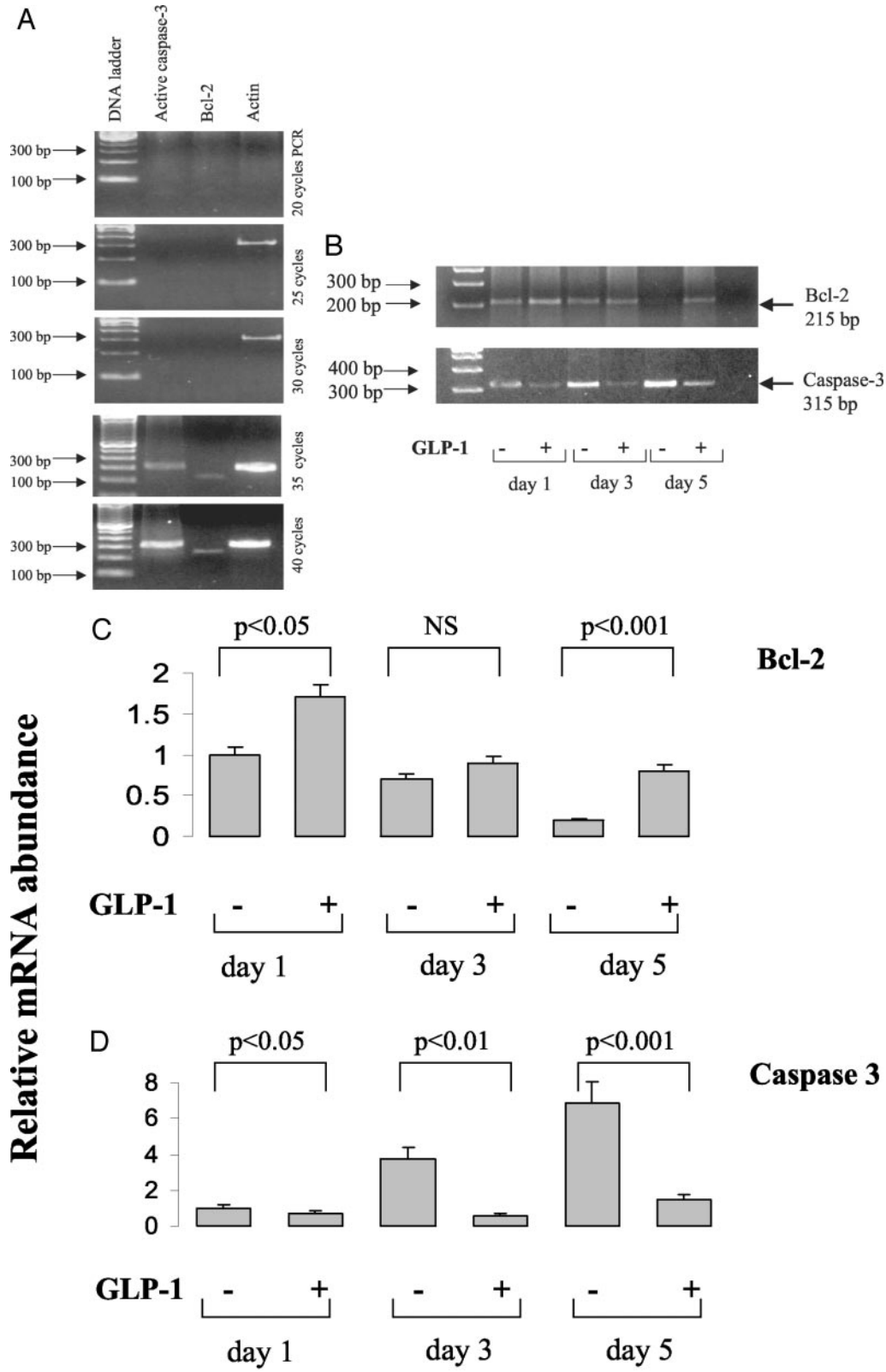

FIG. 6. mRNA levels for bcl-2 and caspase-3. Total RNA from human islets cultured for 1 d in the absence of GLP-1, subjected to reverse transcription and used for PCR-cycle titration to amplify bcl-2, caspase3, and β-actin mRNA. The cDNA was subjected to 20, 25, 30, 35, and 40 cycles of amplification (A). The cDNA prepared from islets cultured for 1, 3, or 5 d in the presence, or absence, of GLP-1 was then subjected to 40 cycles of PCR for bcl-2 and caspase-3 and 30 cycles for β-actin (B). Bar graphs represent the mRNA quantification for bcl-2 and caspase-3 after normalization for β-actin mRNA levels: bcl-2 mRNA quantification (C); and caspase-3 mRNA quantification (D). Statistical significance of the data (treated *vs.* control) was evaluated by Student's *t* test.

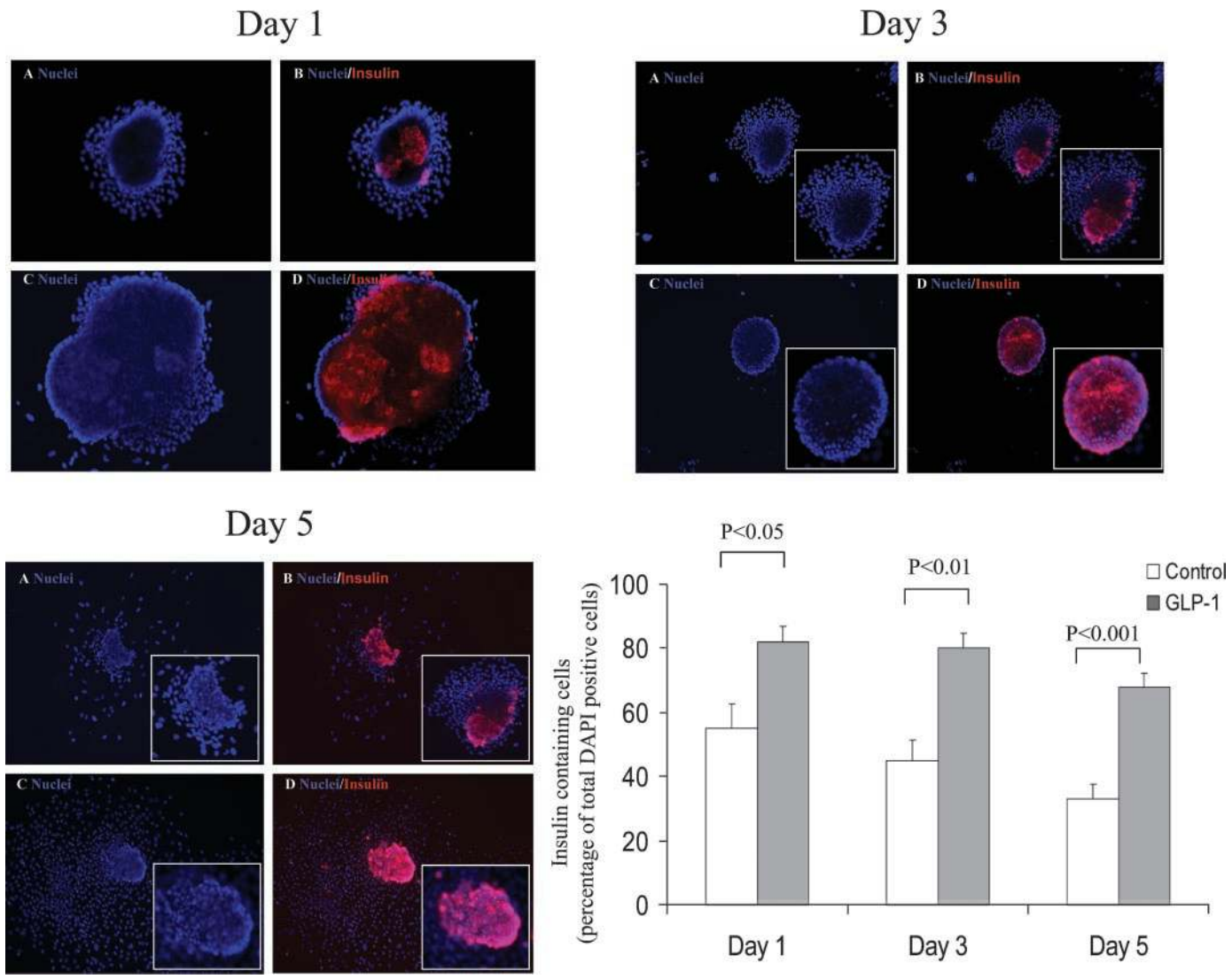

FIG. 7. Immunofluorescence staining for insulin: d 1, 3, and 5. For each individual day, A and B show control islets (×40 and computer-generated further magnification); A, DAPI nuclei staining. B, Overlapping of the nuclei staining and insulin (*red*). C and D, GLP-1-treated islets. C, DAPI nuclei staining. D, Overlapping of the nuclei staining and insulin (*red*). Pictures are representative of islet morphology as observed by culturing human islets from three independent donors. The graph in the *lower left side* represents the percentage of insulin-positive cells in each culture. The data were obtained by the point counting of double-positive cells (insulin and DAPI) divided for the number of DAPI-positive cells. At least 10 islets per each of the individual islet preparation (n = 3) were used to generate these data. Statistical significance of the data (treated *vs.* control) was evaluated by Student's *t* test. Specificity of immunostaining was validating by the lack of fluorescent signal obtained by tissue staining in the absence of the primary antibody.

indicated that GLP-1 and exendin-4 have antiapoptotic properties in addition to their effect on glucose-dependent insulin secretion and the expansion of islet cell mass.

Using Zucker diabetic rats, an animal model of type 2 diabetes in which the onset of the disease occurs when the rate of β-cell apoptosis overcomes the rate of β-cell proliferation, we demonstrated that GLP-1 was capable of restoring near-normal insulin secretion (8). Interestingly, this was observed even when the apoptotic changes in the structure and function of the pancreatic islets had already taken place and hyperglycemia has occurred. In Zucker diabetic rats, apoptotic cells were detected throughout the entire pancreas, including both the exocrine and the endocrine components. Treatment with GLP-1 drastically reduced the number of apoptotic cells. The islets of Langerhans of GLP-1-treated rats had significantly fewer apoptotic nuclei, and the DAPI staining demonstrated that islet cells of animals in this treatment group were healthier and were not subjected to the biological changes leading to cell apoptosis. Double immunostaining for insulin and caspase-3 further supported the evidence for an antiapoptotic action of GLP-1.

Similar data were recently produced in streptozotocin-induced diabetes in mice when treated with exendin-4 (9). In those mice, blood glucose remained significantly lower for weeks after the cessation of the treatment with exendin-4. Conversely, mice with a targeted disruption of the GLP-1 receptor gene exhibited increased apoptosis after streptozotocin administration. Furthermore, exendin-4 directly re-

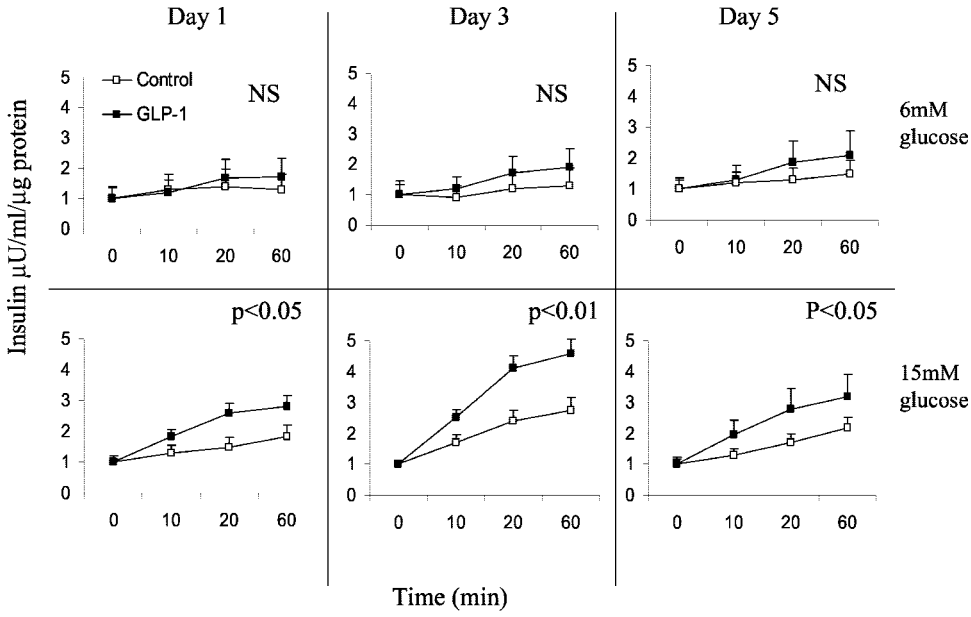

FIG. 8. GLP-1 effect on glucose-dependent insulin secretion. Glucose-dependent secretion of insulin was evaluated after 1, 3, or 5 d from islets cultured in the presence or absence of GLP-1 (10 nM, added every 12 h). Islets were kept in 6 mM glucose until the day of the experiment and then either challenged with 15 mM glucose or maintained in 6 mM glucose. Insulin levels were detected by RIA and normalized for total protein content in the cell pellet. Each experiment was repeated three times, and the data plotted on the graph represent the mean ± SD. Statistical significance of the data were evaluated by ANOVA and indicates the difference between the insulin secretion curves derived from GLP-1-treated *vs.* control islets; *, $P < 0.01$.

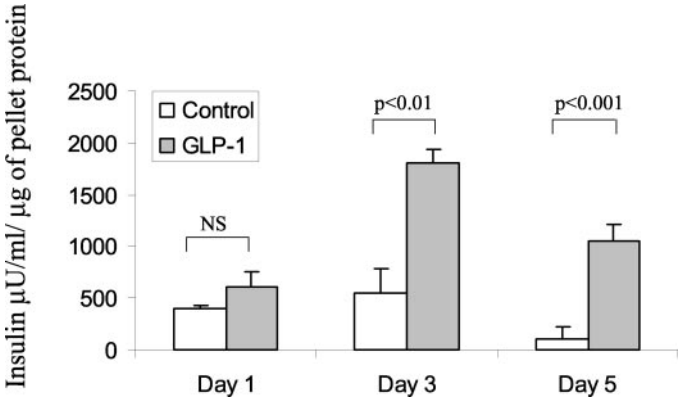

FIG. 9. GLP-1 effect on intracellular insulin content. Cell pellet from islets cultured in the presence or absence of GLP-1 for 1, 3, or 5 d was assayed for insulin content. Cells were cultured in the presence or absence of GLP-1 (10 nM, added every 12 h). Insulin levels were detected by RIA and normalized for total protein content in the cell pellet. Each experiment was repeated three times, and the data plotted on the graph represent the mean ± SD. Statistical significance of the data were evaluated by Student's *t* test.

duced cytokine-induced apoptosis in purified islets β-cells exposed to IL-1β, TNFα, and interferon-γ (9).

We also showed that the antiapoptotic effect of GLP-1 is independent from its glucose-lowering activity, as demonstrated by *in vitro* experiments in which GLP-1 was capable of inhibiting H_2O_2-dependent apoptosis (10). GLP-1 reduced DNA fragmentation and improved cell survival. This was mediated by an increased expression of the antiapoptotic proteins bcl-2 and bcl-xL. GLP-1 also prevented the H_2O_2-dependent cleavage of poly-(ADP-ribose)-polymerase. The antiapoptotic action of GLP-1 was mediated by a cAMP and phosphatidylinositol 3-kinase-dependent signaling pathway. In another study (9), investigators demonstrated that sorted rat β-cells, incubated with exendin-4 and a combination of cytokines, which included IL-1β, TNFα, and interferon γ for 18 h, had an apoptosis rate decreased by 44%, compared with controls. Studies using baby hamster kidney fibroblasts transfected with the GLP-1 receptor demonstrated that exendin-4 had a protective effect on cells exposed to the proapoptotic agent cycloheximide (9). This antiapoptotic action of exendin-4 was associated with a reduced expression of caspase-3, caspase-8, and caspase-9 and a decrease in the release of cytochrome *c* from the mitochondria. The cells also had preserved levels of protein kinase B/Akt and β-catenin, a caspase-3 substrate. The combination of these effects resulted in an increased cell viability over time.

In the present study, we investigated whether the antiapoptotic action of GLP-1 could be beneficial to the preservation of mass and function of freshly isolated human islets. We demonstrated that GLP-1 delayed the morphological changes that occurred in human islets in culture, as indicated by a longer-lasting preservation of their 3-D structure, with maintenance of the noncellular membrane that surrounds healthy human islets. GLP-1 promoted a time-dependent increase in the expression of the antiapoptotic protein bcl-2 and a down-regulation of the intracellular levels of the active form of caspase-3. A similar effect was observed at the mRNA level for bcl-2 and caspase-3. We also showed that by improving cell viability we were able to show a significant amelioration of islet cell function. Indeed, GLP-1-treated human islets contained more insulin and were capable of a greater glucose-dependent insulin secretion.

In this short-term experiment (the islets were cultured up to 5 d), we did not observe an increase in the size or number of islets in response to GLP-1. The lager size of the islets cultured in the presence of GLP-1 was more the result of a decrease in islet mass occurring in the control cultures. GLP-1-treated islets appeared larger than control by default because they maintain their integrity and did not lose cells over time.

Although the present studies were not designed to investigate whether GLP-1 was capable of inducing the proliferation of mature human β-cells, we cannot rule out that this process (in addition to the inhibition of apoptosis) may also take place when islets are exposed to GLP-1. Experiments

specifically designed to address this question may be necessary to fully elucidate the potential effect of GLP-1 in regulating human islets cell mass.

Our and other authors' results suggest that GLP-1 is not only a growth factor for β-cells (7, 23–27) but also a powerful antiapoptotic agent (8–10) contributing to the observed increase in islet cell mass in previous *in vivo* models. These observations may have important clinical and therapeutic implications. Indeed, GLP-1 could be viewed as an important agent to be used when isolating and culturing human islets before transplantation in human subjects with type 1 diabetes. We propose that GLP-1 could improve mass and function of islet cells and perhaps limit the frequent requirement of more than one donor to render recipient subjects euglycemic. Furthermore, because GLP-1 is being considered for the treatment of type 2 diabetes, the identification of its antiapoptotic properties may better define the indication for its use in subjects at early stages of the disease when restoration of a normal islet mass delays or perhaps prevents the onset of diabetes.

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