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Glucagon-Like Peptide-1 Promotes Islet Cell Growth and Inhibits Apoptosis in Zucker Diabetic Rats

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A constant remodeling of islet cell mass mediated by proliferative and apoptotic stimuli ensures a dynamic response to a changing demand for insulin. In this study, we investigated the effect of glucagon-like peptide-1 (GLP-1) in Zucker diabetic rats, an animal model in which the onset of diabetes occurs when the proliferative potential and the rate of β-cell apoptosis no longer compensate for the increased demand for insulin. We subjected diabetic rats to a 2-d infusion of GLP-1 and tested their response to an ip glucose tolerance test. GLP-1 produced a significant increase of insulin secretion, which was paralleled by a decrease in plasma glucose levels ($P < 0.001$ and $P < 0.01$, respectively). Four days after the removal of the infusion pumps, rats were killed and the pancreas harvested to study the mechanism by which GLP-1 ameliorated glucose tolerance. *Ex vivo* immunostaining with the marker of cell proliferation, Ki-67, showed that the metabolic changes observed in rats treated with GLP-1 were associated with an increase in cell proliferation of the endocrine and exocrine component of the pancreas. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining, a marker of cellular apoptosis, indicated a reduction of apoptotic cells within the islet as well in the exocrine pancreas in GLP-1-treated rats. Double immunostaining for the apoptotic marker caspase-3 and for insulin showed a significant reduction of caspase-3 expression and an increase in insulin content in GLP-1-treated animals. Finally, staining of pancreatic sections with the nuclear dye 4,6-Diaminidino-2-phenyl-dihydrochloride demonstrated a marked reduction of fragmented nuclei in the islet cells of rats treated with GLP-1. Our findings provide evidence that the beneficial effects of GLP-1 in Zucker diabetic rats is mediated by an increase in islet cell proliferation and a decrease of cellular apoptosis. (*Endocrinology* 143: 4397–4408, 2002)

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is a 300-amino-acid peptide secreted from the L-cells of the intestinal epithelium in response to food. GLP-1 is formed as a result of proteolytic cleavage of proglucagon (1, 2) and is the most potent insulinotropic hormone known (3, 4). GLP-1 action is mediated by binding to a cell surface receptor, which belongs to the secretin/glucagon superfamily of receptors that are coupled to heterotrimeric G proteins. Binding of GLP-1 to its receptor stimulates cAMP formation and a raise in intracellular Ca^{2+} (5, 6). Although the endogenous postprandial levels of intact and biological active GLP-1 has been shown to be reduced in subjects with diabetes (7), the insulinotropic activity of exogenously administered GLP-1 has been shown to be retained, even many years after the onset of hyperglycemia (8). Indeed, following administration of intravenous GLP-1, the insulin secretory response in nondiabetic and diabetic subjects is remarkably similar (8). When administered to subjects with type 2 diabetes, whose fasting blood glucose was poorly controlled on diet and sulfonylurea therapy, GLP-1 normalizes their fasting glucose levels (9). These observations suggest that even if β-cells of the pancreas no longer respond to sulfonylureas, GLP-1 therapy may still be an option for the treatment for type 2 diabetes.

In glucose-intolerant Wistar rats, the mechanisms by which GLP-1 reverses the age-dependent β-cell abnormalities include a transcription activation of insulin, glucose transporter-2, and glucokinase genes (10). This is associated with an expansion of β-cell mass via islet cell neogenesis (11). In the present study, we investigated the capability of GLP-1 to improve glucose control in a model of overt diabetes, the Zucker diabetic rat. Inbred Zucker diabetic fatty (ZDF/GmiTM-fa/fa) rats develop diabetes around 10 wk of age after a prediabetic period during which obesity and insulin resistance are present, but fasting blood glucose concentration is normal (12, 13). Compared with lean control rats from a partially outbred strain that develops insulin resistance but not diabetes, ZDF rats show an increased islet cell replication rate, and the clinical onset of diabetes has been demonstrated to depend on an increase of islet cell apoptosis (14).

In the present study, we provide evidence that the antidiabetic action of GLP-1, in Zucker rats, depends upon its proproliferative action and inhibition of islet cell apoptosis.

Materials and Methods

Animals

Twelve-week-old male ZDF rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained on standard laboratory chow under a 12-h light/12-h dark schedule. They were given

Abbreviations: AUC, Area under the curve; DAPI, 4,6-diaminidino-2-phenyl-dihydrochloride; GLP-1, glucagon-like peptide-1; IPGTT, ip glucose tolerance test; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-digoxigenin nick-end labeling; ZDF, Zucker diabetic fatty (rat).

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free access to food and water and fasted only for the night before the glucose tolerance test. All institutional guidelines for care and use of animals were followed. All ZDF rats were diabetic at the beginning of the study, as indicated by their fasting glucose levels. A total of eight animals per treatment group were employed for the study. Although all animals (treated and controls) were subjected to glucose tolerance tests, a minimum of four animals per treatment group were used for any of the immunohistological and morphometric study hereafter described.

Experimental design

ZDF rats were subjected to a 2-d continuous infusion of human recombinant GLP-1 (Bachem, King of Prussia, PA), (30 pmol/kg·min) or saline (n = 8 per treatment group). The infusion was performed using an Alzet microosmotic pump (Alza Corp., Minneapolis, MN) implanted in the interscapular region. Before the surgical implantation of the pump, the rats were anesthetized using 45 mg/kg ketamine (Phoenix Scientific, Inc., St. Joseph, MO) and 4.5 mg/kg xylazine (Loyd Laboratories, Shenandoah, IA). The infusion pumps were removed at the end of the infusion. Four days after the removal of the pumps (corresponding to d 6 from the beginning of the experiment, counting as d 1 the day of the beginning of the infusion), they were subjected to an ip glucose tolerance test (IPGTT; 1 g glucose/kg body weight) after an overnight fast. Blood samples were collected by tail tip amputation at −20, 2, 15, 30, 45, 60, 90, and 120 min from the beginning of the glucose injection. This procedure allowed for blood collection without cutting the skeletal component of the tail and did not require the catheterization of a blood vessel. Blood samples were then assayed for glucose and insulin levels. Rats were killed 1 d after the IPGTT (d 7) and pancreata were harvested and fixed in paraffin for histological studies. Dose and duration of GLP-1 infusion were based on a previously published study from our laboratory (15). Fasting plasma GLP-1 levels were measured in GLP-1-infused and control rats the day before the beginning of the infusion, 30 min after the end of the infusion, and the day on which the animals were killed for histology studies.

GLP-1-dependent proliferation of pancreatic β-cells

To assess the effect of GLP-1 on the proliferation of β-cells, pancreatic tissue sections were deparaffinized and stained for the presence of the Ki-67 antigen (a marker of cell mitosis) and insulin. Briefly, the process required immersing the tissue containing slides in three baths of xylene. They were then rehydrated in two baths of 100% ethanol, one bath of 90% ethanol, and two baths of 70% ethanol. Finally, the sections were treated with proteinase-K (Sigma, St. Louis, MO), 20 μg/ml, for 6 min at room temperature and washed in PBS (Sigma) for 5 min.

A double staining for the nuclear proliferative marker Ki-67 and insulin was then performed. All steps were carried out at room temperature unless otherwise described. The sections were first incubated with normal horse serum for 30 min. A Ki-67 mouse antirat monoclonal antibody (Novocastra, New Castle Upon Tyne, UK), diluted 1:50 in PBS, was incubated with the sections overnight at 4 C. After several washes, the secondary antibody, an affinity-purified antimouse and rhodamine-conjugated IgG (Chemicon, Temecula, CA), was added at the dilution of 1:50 for 1 h. For insulin immunostaining, the sections were incubated for 1 h with a polyclonal guinea pig antirat insulin antibody (DAKO Corp., Carpinteria, CA), diluted 1:50. The sections were then subjected to two washes in PBS before the addition of a fluorescein isothiocyanate-conjugated rabbit antiguinea pig secondary antibody (DAKO Corp.), diluted 1:40, and incubated for 1 h.

The tissue slides were analyzed with a BH-2 microscope (Olympus Corp., Melville, NY) using a fluorescein standard filter set (520 ± 20 nm) for insulin staining and a rhodamine filter (>620 nm) for Ki-67 staining.

GLP-1-dependent effect on β-cell apoptosis

Terminal deoxynucleotide transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL). The TUNEL assay was performed using a DeadEnd colorimetric apoptotic detection system (Promega Corp., Madison, WI). All incubations were performed at room temperature, unless otherwise specified. The paraffin-embedded pancreas sections were deparaffinized and rehydrated as described above. The slides were then incubated in 0.85% NaCl (Sigma) for 5 min and washed in PBS for 5 min. They were then fixed in 4% formaldehyde (J. T. Baker, Phillipsburg, NJ) for 15 min and washed twice in PBS. The tissues were covered with 100 μl proteinase-K (20 μg/ml) and incubated for 30 min. Immersing the slides in PBS for 5 min stopped the protein digestion by proteinase-K. The slides were then transferred into a jar containing 4% formaldehyde and incubated for 5 min. After one wash in PBS, the slides were incubated for 10 min in equilibration buffer provided with the detection kit and then covered with a solution containing a biotinylated nucleotide mix and the TdT-terminal transferase. The incubation was performed at 37 C for 1 h. Immersing the slides in 2 × saline sodium citrate for 15 min then terminated the reaction. After three washes in PBS, the sections were incubated with 0.3% hydrogen peroxide for 5 min to block endogenous peroxide. Streptavidin horseradish peroxidase solution (Promega Corp.), diluted 1:500 in PBS, was added and incubated for 30 min. After washing twice in PBS, the colorimetric reaction was developed by using the diamino-benzidine reagents and counterstained with eosin. The tissue slides were analyzed by light microscopy using a BH-2 microscope (Olympus Corp.).

Double immunofluorescence for insulin and caspase-3. Pancreatic sections obtained from GLP-1-treated and control rats were also subjected to a double immunostaining for caspase-3 and insulin. After deparaffinization, the sections were incubated with a polyclonal rabbit anticaspase-3 antibody (BD PharMingen, Lexington, KY) diluted 1:500 in PBS and with a polyclonal guinea pig antiinsulin antibody (DAKO Corp.), diluted 1:50. The incubation was performed at 4 C overnight. After several washes in PBS, an antirabbit fluorescein-conjugated antibody (Chemicon) diluted 1:100, and an antiguinea pig rhodamine-conjugated antibody (DAKO Corp.), diluted 1:40, were incubated for 1 h at room temperature.

4,6-Diaminidino-2-phenyl-dihydrochloride (DAPI) staining. Pancreatic sections were fixed as described above and rinsed in phosphate buffer solution (1 M PBS, pH 7.4) before the addition of the mounting medium containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA), and the slides were examined using a digital camera (model KX2E, Apogee, Aubum, CA) attached to the BH-2 microscope (Olympus Corp.) and processed with an Image-Pro computer program (Media Cybernetics, L.P., Carlsbad, CA).

Measurement of islet cell mass, β-cell proliferation, and β-cell apoptosis

Quantification of islet cell mass, β-cell proliferation, and apoptosis was performed by point-counting morphometry of insulin-immunostained pancreatic sections, with minor adaptation to the method described by Weibel (16) and later applied by Bonner-Weir and colleagues (11, 14, 17). For this study, pancreas sections obtained from four ZDF rats treated with GLP-1 and four control ZDF rats infused solely with saline solution were analyzed. Each pancreas was divided into two blocks, and for each block various independent sections were analyzed to acquire approximately 200 fields per pancreas for final quantification of mass, proliferation, and apoptosis. The slides were examined at the final magnification of ×400, using a digital camera (model KX2E, Apogee) attached to the BH-2 microscope (Olympus Corp.) and processed with an Image-Pro computer program (Media Cybernetics).

For assessment of β-cell mass, a color monitor with a dotted transparent overlay was used for point counting. In nonoverlapping fields, the number of intercepts over β-cell, endocrine non-β-cell, and exocrine tissue was determined.

For assessment of β-cell proliferation, pancreatic sections were stained for both Ki-67 and insulin. The number of cells that costained for both insulin and Ki-67 was divided by the total number of insulin-positive cells per each field to obtain the percentage of actively dividing β-cell.

To determine the percentage of islet β-cells undergoing apoptosis, pancreatic sections were stained with the nuclear dye DAPI and an antibody-recognizing insulin. The DAPI staining allows visualizing fragmented/damaged nuclei of cells that have initiated the process leading to apoptosis. The number of insulin-positive cells with fragmented nuclei was then divided for the total number of insulin-positive cells per each field to determine the percentage of apoptotic β-cells.

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TABLE 1. General physiological characteristics of Zucker diabetic rats used for the study

	GLP-treated (n = 8) 12 wk, male	Controls (n = 8) 12 wk, male	Statistical significance
Body weight (g, d-0)	487 ± 37	476 ± 41	n.s.
Body weight (g, d-7)	421 ± 23	458 ± 62	$P < 0.01$
Pancreatic weight (mg, d-7)	1.20 ± 0.03	1.18 ± 0.05	n.s.
AUC for glucose (d-6)	65% of controls	100%	$P < 0.05$
AUC for insulin (d-6)	240% of controls	100%	$P < 0.001$
GLP-1 (pM, d-0)	22.3 ± 3.6	20.8 ± 3.0	n.s.
GLP-1 (pM, d-3)	63.7 ± 6.9	19.1 ± 4.2	$P < 0.001$
GLP-1 (pM, d-6)	20.1 ± 4.2	23.2 ± 2.6	n.s.

d-0 refers to the day before the GLP-1 infusion, whereas d-3, d-6, or d-7 refer to number of days from the beginning of the GLP-1 infusion. Statistical significance of the data was evaluated by unpaired Student's t test.

All morphometric analyses were performed in blind by one observer (L.F.).

Plasma assays

The plasma levels of insulin and the active form of GLP-1 were measured by RIA (Linco Research, Inc., St. Charles, MA). Plasma glucose was measured by the glucose oxidase method using a CCX Spectrum (Abbott, Irving, TX). The area under the curve (AUC) for insulin and glucose was calculated according to the trapezoid rule.

Statistical analysis

The data were expressed as mean ± SE. Significance of the data was evaluated by unpaired t test. One-way ANOVA was used to evaluate statistical significance when more than two data points were analyzed. Use of statistical analyses by unpaired t test or ANOVA is explicitly identified in the text or figure legends.

Results

Plasma GLP-1 levels

Plasma GLP-1 levels were measured before the day of the implantation of the infusion pumps, 30 min after the termination of the infusion, and immediately before the glucose tolerance test (on d 6 of the experiment). All blood sampling was performed after an overnight fast. No statistically significant difference in the plasma level of GLP-1 was observed among rats tested before the beginning of the treatment. A statistically greater level of GLP-1 was detected in GLP-1-treated rats, compared with controls, when the plasma was collected 30 min after removal of the infusion pumps (Table 1). The two treatment groups reverted to approximately equal GLP-1 levels by the time of the measurement on d 6, indicating that all exogenous GLP-1 was metabolized by time the time of the glucose tolerance test.

Glucose tolerance in ZDF rats

The infusion of GLP-1 to diabetic rats had a significant positive effect on both glucose excursion and insulin secretion, as demonstrated by an IPGTT performed 4 d after the end of the treatment (Fig. 1). This occurred by increasing and decreasing the AUC for insulin and glucose, respectively, within the first 30 min from the beginning of the glucose infusion. GLP-1 partially restored the acute phase secretion of insulin, characteristically lost in ZDF rats, and enhanced the overall insulin secretory activity in response to glucose. The AUC for insulin, calculated from −20 min to 30 min, showed that GLP-1-treated rats had a 1.7-fold increase in insulin levels ($P < 0.001$, Table 1). GLP-1 also lowered plasma

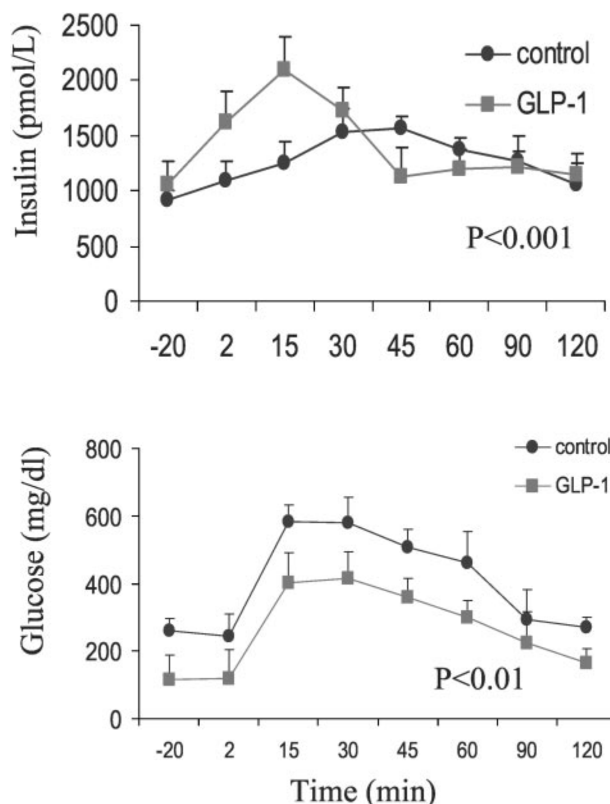

FIG. 1. Insulin and glucose secretion. Insulin *(top panel)* and glucose *(bottom panel)* levels were evaluated by subjecting ZDF rats to an IPGTT (1 g/kg body weight), performed 4 d after a 48-h infusion with GLP-1 (30 pmol/kg·min) or saline solution. The data were obtained from eight different animals per treatment group. Statistical significance was evaluated by ANOVA from time point between −20 and 30 min.

glucose levels after the initial spike, occurring within 15 min from the beginning of the test ($P < 0.01$, GLP-1 *vs.* saline-treated rats), with a return to pre-IPGTT values within 90 min (Fig. 1). The AUC for glucose was 20% lower in GLP-1-treated rats, compared with controls ($P < 0.01$, Table 1).

Islet cell morphology

Treatment with GLP-1 induced a marked increased in the size of the preexisting islets as well as the formation of new isletlike aggregates of insulin-positive cells. In GLP-1-treated ZDF rats, the diameter of large-sized islets was 1.3- to 1.5-fold

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greater than those observed in controls (Fig. 2). Although in controls some large islets reached the diameter of 15,000–18,000 μm^2, the largest islet in the GLP-1-treated group reached 20,000–25,000 μm^2. In GLP-1-treated rats, there was also an enhanced irregularity of the margins of those large islets, with the presence of an extensive branching out of cells from the periphery of the islets. This budding of cells appeared as developing branches of cells reaching and connecting the islets to neighboring ductal structures, although, less frequently, they extended toward the exocrine parenchyma (Fig. 2).

We also observed extraislet aggregates of insulin-positive cells, present after treatment with GLP-1, which were characteristically formed by less than 10 cells and were scattered throughout the entire pancreas. Those clusters of cells did not show any obvious structural connection with preexisting large or medium-sized islets or with ductal structures. These smaller islets (<100 μm^2) had margins characteristically regular and homogeneous, with the absence of invaginations (Fig. 2). Very few insulin-positive cell aggregates were also occasionally observed in control ZDF rats.

A third group of insulin-positive cells was represented by cells that appeared wrapped around small-sized ductal cells, often connecting more than one duct, and forming a netlike structure of irregular shape (Fig. 2). Those insulin-positive cells were observed exclusively in the proximity of small-

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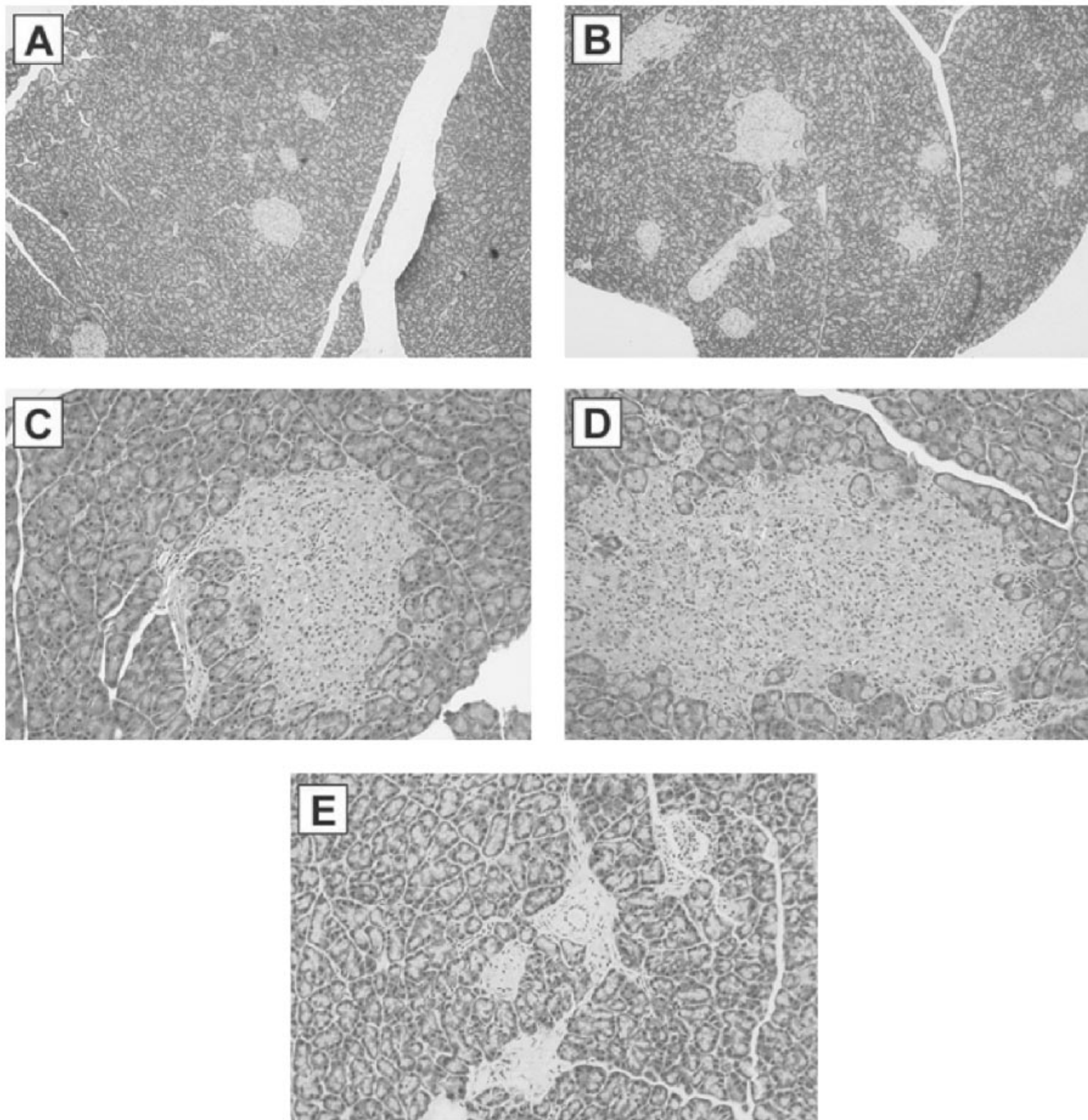

FIG. 2. Islets cell morphology. Pancreatic sections stained with hematoxylin and eosin. Control (A) and GLP-1-treated ZDF (B); ×200 magnification. C and D, Detail of a representative individual islet from control (C) and GLP-1-treated (D) rats, ×400 magnification. E, ×800 magnification of a pancreatic section from a GLP-1-treated rat showing a detail of islet cells budding from pancreatic ducts. Pictures are representative hematoxylin and eosin staining of pancreatic sections of at least five animals per treatment group. At least five sections per each individual pancreas were studied.

sized ducts; no such aggregates of cells were observed in medium- or large-sized ducts. They were characteristically more abundant in GLP-1-treated rats than in controls.

GLP-1-dependent cell proliferation

Immunostaining for the Ki-67 antigen, a nuclear marker of cell proliferation, demonstrated the presence of actively dividing cells both in controls and in GLP-1-treated rats. Ki-67-positive cells were present within the endocrine as well as the exocrine component of the pancreatic parenchyma. In GLP-1-treated animals, the number of Ki-67-positive cells was such to suggest a greater cell proliferation, compared with controls (Fig. 3).

In addition to a quantitative difference in the number of replicating cells, there was a qualitative difference in their appearance in the two treatment groups. Although in the control group the Ki-67-positive cells appeared to be randomly distributed among the population of insulin-secreting cells (Fig. 3, A and B), their pattern of distribution in GLP-1-treated rats showed a much greater variability. Two common presentations of Ki-67-positive cells were observed in animals exposed to GLP-1: sparse, individual cells within the islets of Langerhans (Fig. 3, C and D) and aggregates of isletlike structures that were insulin negative and sparse within the exocrine parenchyma (Fig. 3, G and H). The presence of sparse/scattered Ki-67-positive cells within the islets was characteristic of islet of very large size (Fig. 3, C and D), and it was only rarely observed in small aggregates of

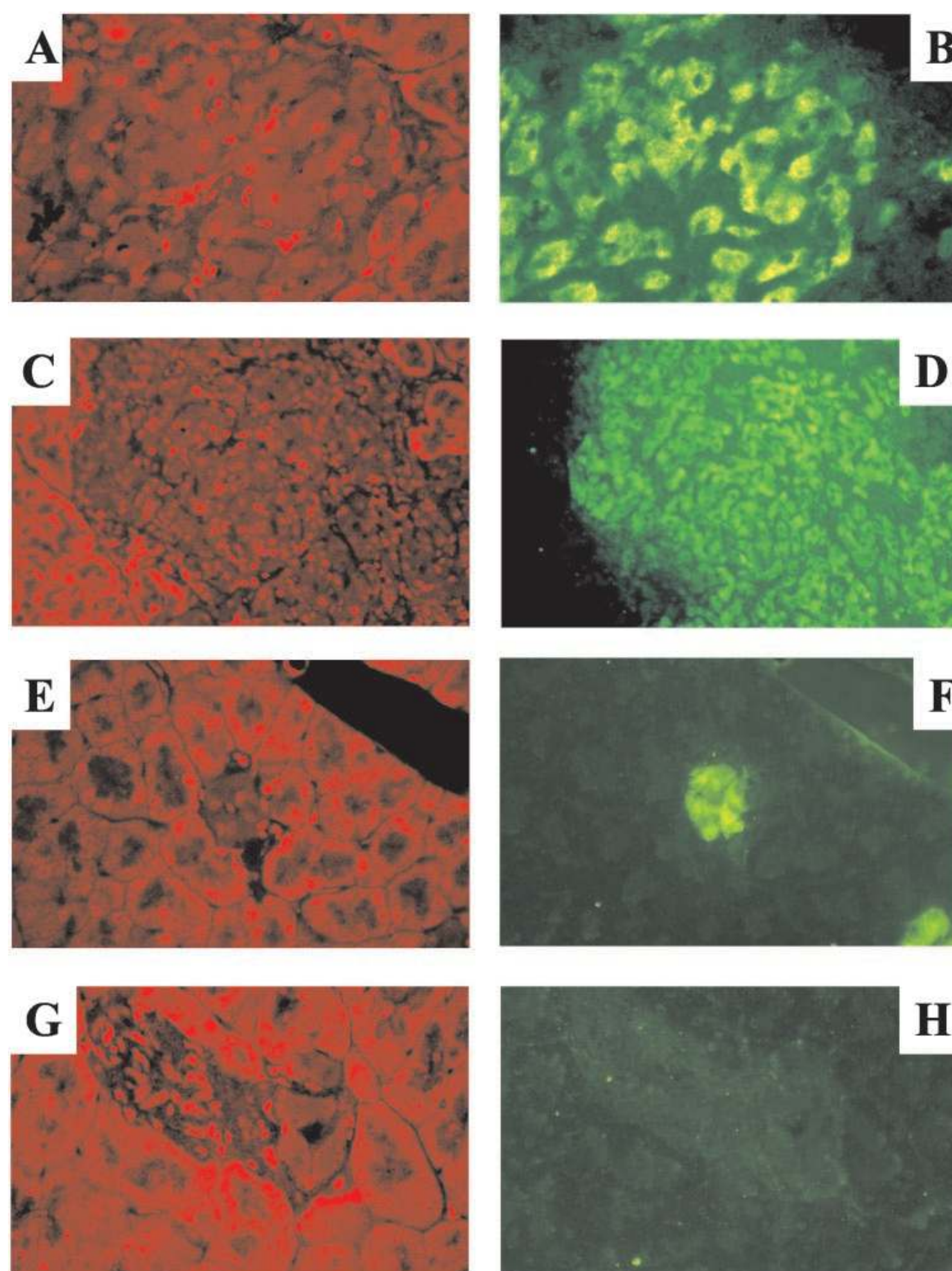

FIG. 3. GLP-1-dependent cell proliferation: double-immunostaining Ki-67 *(red)* and insulin *(green)*. A, Ki-67 staining in control pancreas of a Zucker rat; detail of a large islet. B, Insulin staining of the same islet. C, Ki-67 staining in GLP-1-treated rats; detail of a large islet. D, Insulin staining of the same islet. E, Ki-67 staining of a small islet from the pancreas of a control rat. F, Insulin staining of the same islet. G, Ki-67 of a pancreatic section from a GLP-1-treated rat; aggregates of Ki-67-positive cells. H, Insulin staining of the same area. Staining for Ki-67 shows a characteristic nuclear distribution, and insulin is detected exclusively in the cell cytoplasm. Pictures are representative of double staining for Ki-67 and insulin of pancreatic sections of at least five animals per treatment group. At least five sections per each individual pancreas were studied.

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insulin-positive cells. Although in the control group, the few cell isletlike aggregates that were observed in the exocrine parenchyma always contained insulin (Fig. 3, E and F), in the GLP-1-treated group, we observed aggregates of cells that were positive for Ki-67 but negative for insulin (Fig. 3, G and H). This latter group of clusters of proliferating cells was never observed in the control group.

In addition to those Ki-67-positive cells that appeared as clusters of few cells within the exocrine parenchyma, we observed that similar aggregates of dividing cells could also be detected within the limits of many islets (Fig. 4). This was more frequently observed in medium- and small-sized islets and was never observed in the control group. The diving cells within those islets were distinct from the differentiated insulin-secreting cells. As shown in Fig. 4, the aggregates of Ki-67-positive cells were often at the margins of differentiated β-cells (Fig. 4, D–I) and even when present in the core region of an islet (Fig. 4, A–C), they never expressed insulin.

The tendency of forming aggregates of mitotic cells was never observed in the saline-infused controls. Although Ki-67-positive cells were clearly detected in control rats, they invariably appeared as scattered, isolated, single cells that were present within all three cell compartments of the pancreas: endocrine, exocrine, and ductal. Finally, the clusters of Ki-67-positive cells detected in the exocrine parenchyma were made up of cells that appeared larger than the surrounding cells because of the presence of both larger nuclei and wider and more translucent cytoplasmic components.

GLP-1-dependent inhibition of cell apoptosis

Using the TUNEL assay, we observed a greater number of apoptotic cells in the pancreatic sections of untreated ZDF rats, compared with those collected from the GLP-1-treated group (Fig. 5). In the control group, we detected several aggregates of apoptotic cells; they were more characteristically present in the exocrine parenchyma and frequently in

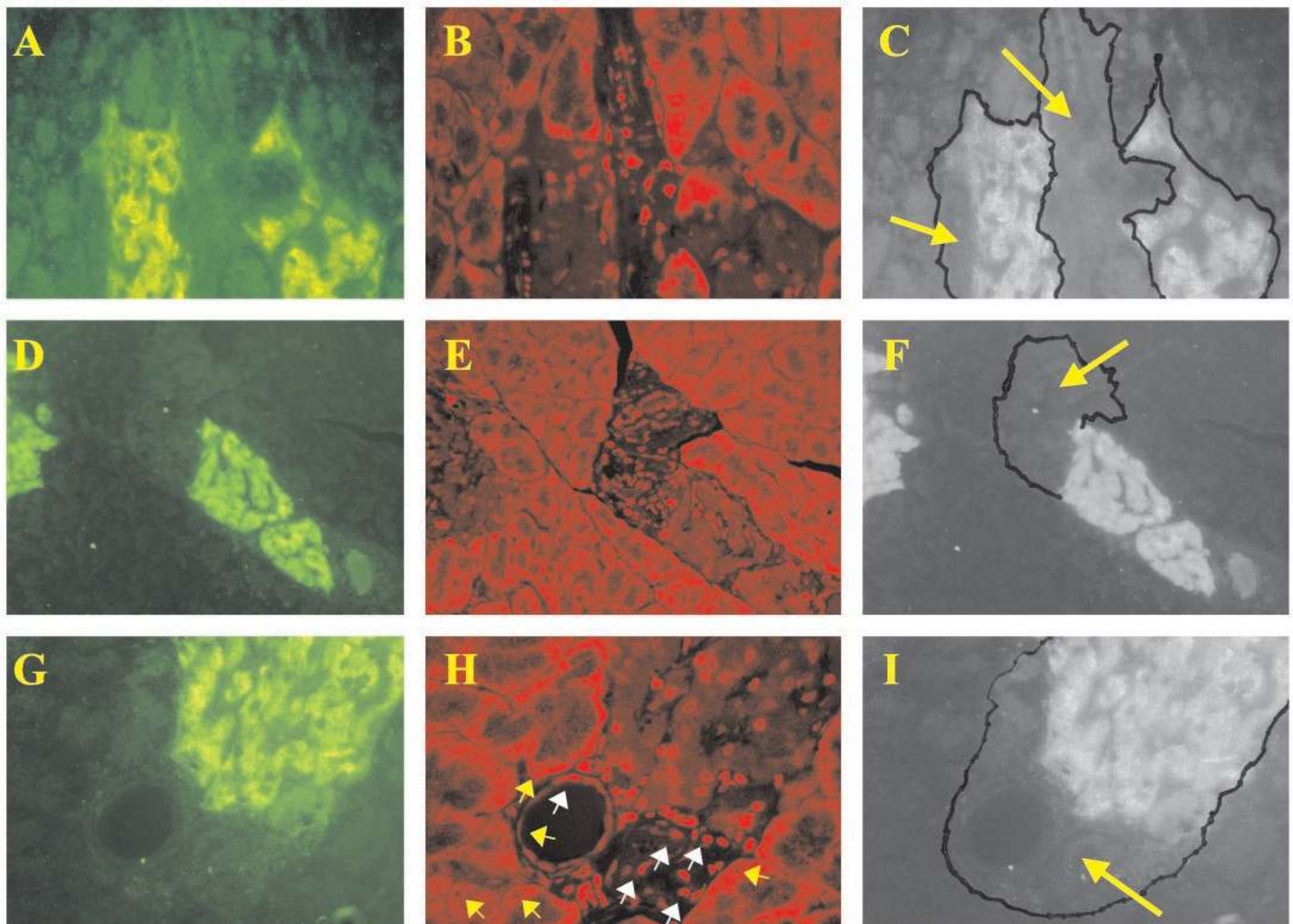

FIG. 4. Cell distribution of insulin and Ki-67 in an individual islet of Langerhans. Double-immunostaining Ki-67 *(red)* and insulin *(green)*. Three examples of individual islets of Langerhans from GLP-1-treated ZDF rats. A, D, and G, Staining for insulin. B, E, and H, Staining for Ki-67 antigen. C, F, and I show aggregates of Ki-67-positive cells present within the limit of three different islets. Staining for Ki-67 shows a characteristic nuclear distribution, and insulin is detected exclusively in the cell cytoplasm. The *white arrows* in H show cells with a positive nuclear staining for Ki-67, and the *yellow arrows* indicate cells that were not recognized by the Ki-67 antibody. The *arrows* in C, F, and I indicate islet regions that are Ki-67 positive but insulin negative. The islet magnification was further enlarged by computer modification to ×1000.

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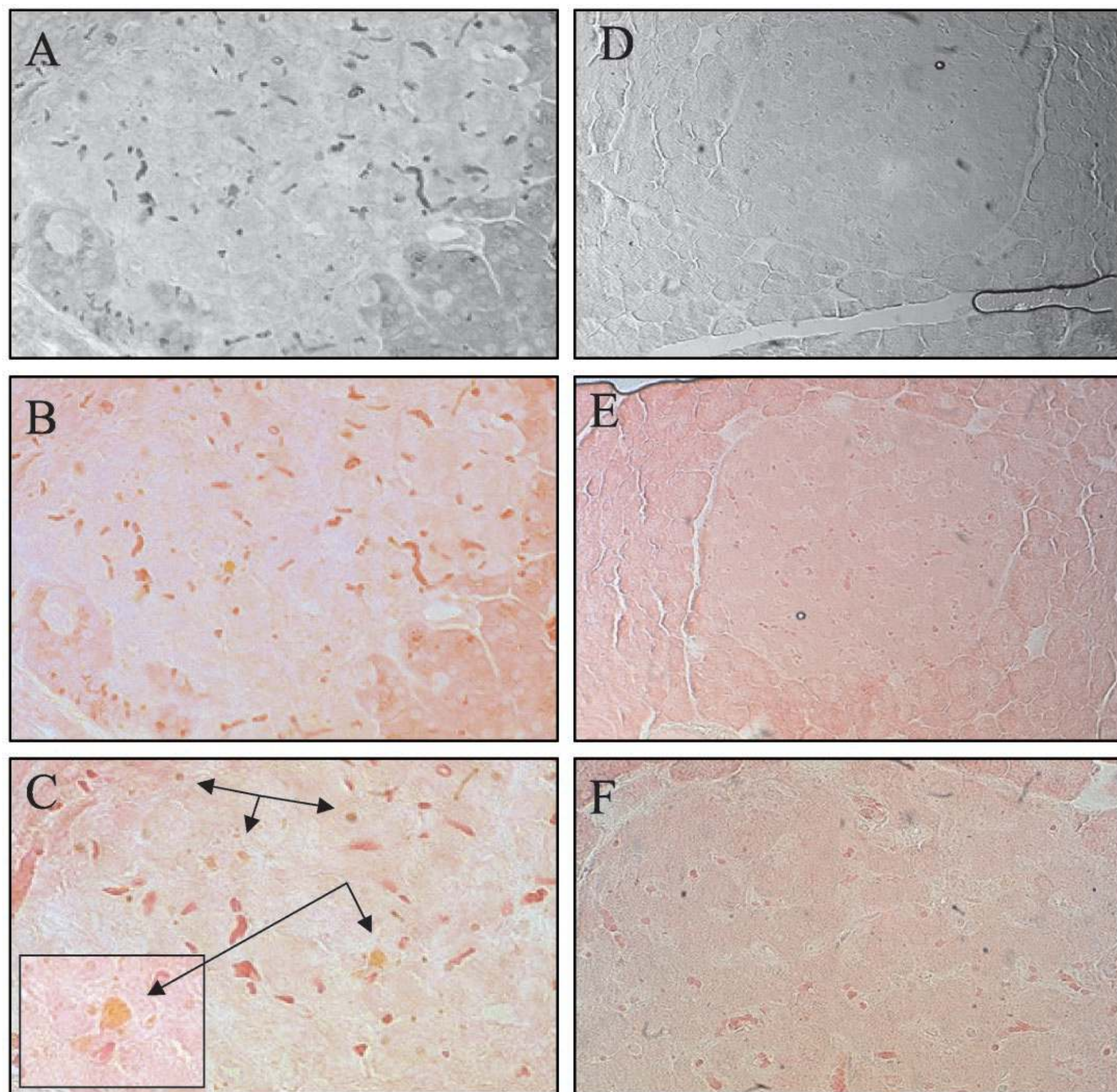

FIG. 5. Detection of cell apoptosis by TUNEL assay. A, B, and C, Saline-infused ZDF rats. A and B, ×20 Magnification; C, ×40 magnification and detail of apoptotic nuclei at ×100. D, E, and F, GLP-1-treated ZDF rats. D and E, ×20 Magnification; F, ×40 magnification. The *arrows* indicate examples of apoptotic areas. Pictures are representative of TUNEL assay of pancreatic sections obtained from at least five animals per treatment group. At least five sections per each individual pancreas were studied.

close proximity of several islets. There were also isolated fragmented nuclear apoptotic cells localized within the islets (Fig. 5).

In GLP-1-treated ZDF rats, unlike the control group, we did not observe any clusters of apoptotic cells in the pancreatic sections examined. There were also fewer isolated apoptotic nuclei scattered throughout the exocrine pancreas, and the islets were virtually free of apoptotic cells. GLP-1 appeared to promote a general improvement of the islet morphology, characterized by a reorganization of the islet structure with a marked reduction of noncellular areas (Fig. 5).

Consistent with the data derived from the TUNEL assay, the double staining for caspase-3 and insulin showed a significant reduction of the caspase-3 expression in GLP-1-treated rats, compared with controls (Fig. 6, A and B). These changes were associated with the presence of a greater number of insulin-containing cells in the context of morphologically more organized islets. As observed for the endocrine

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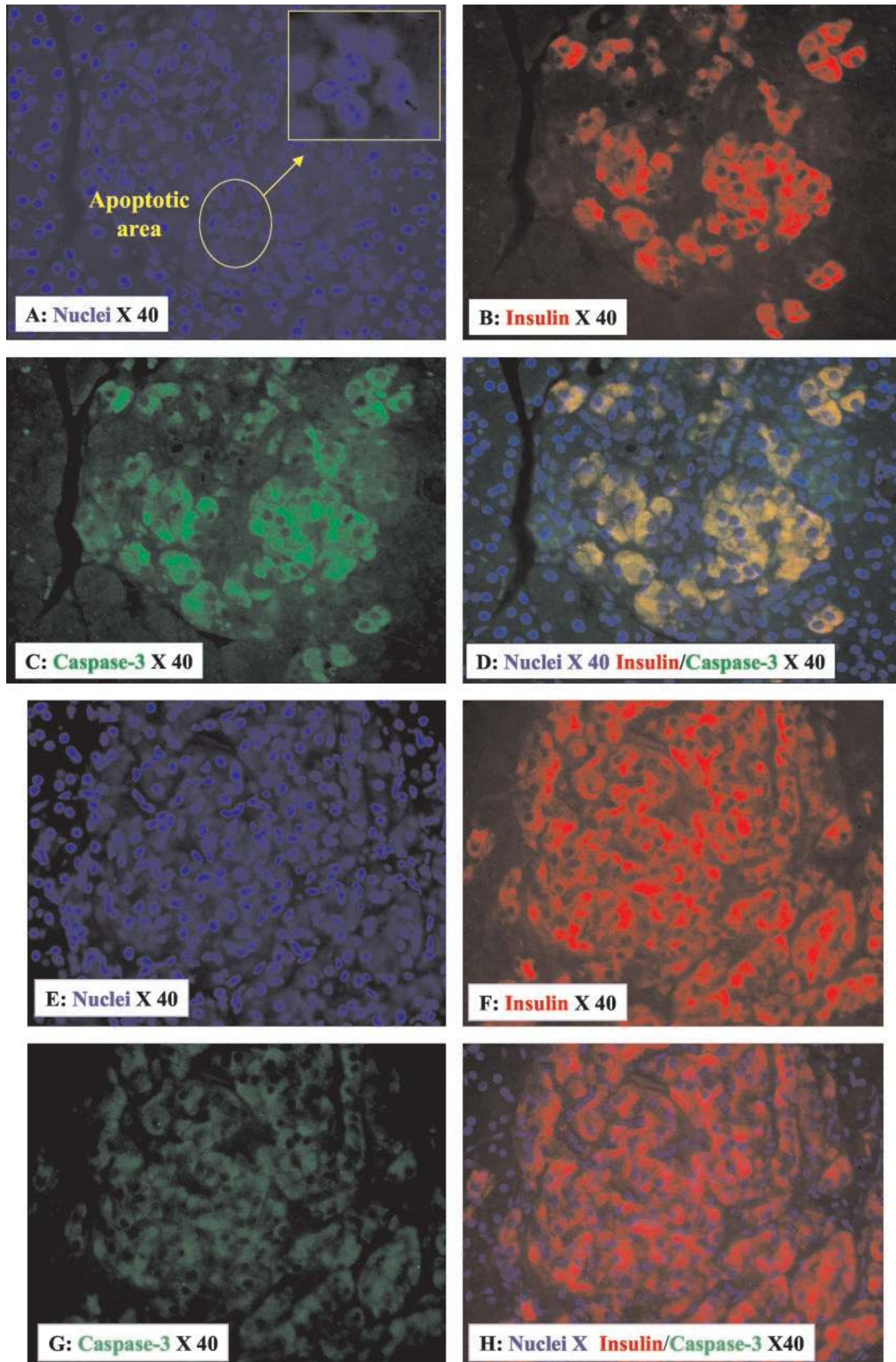

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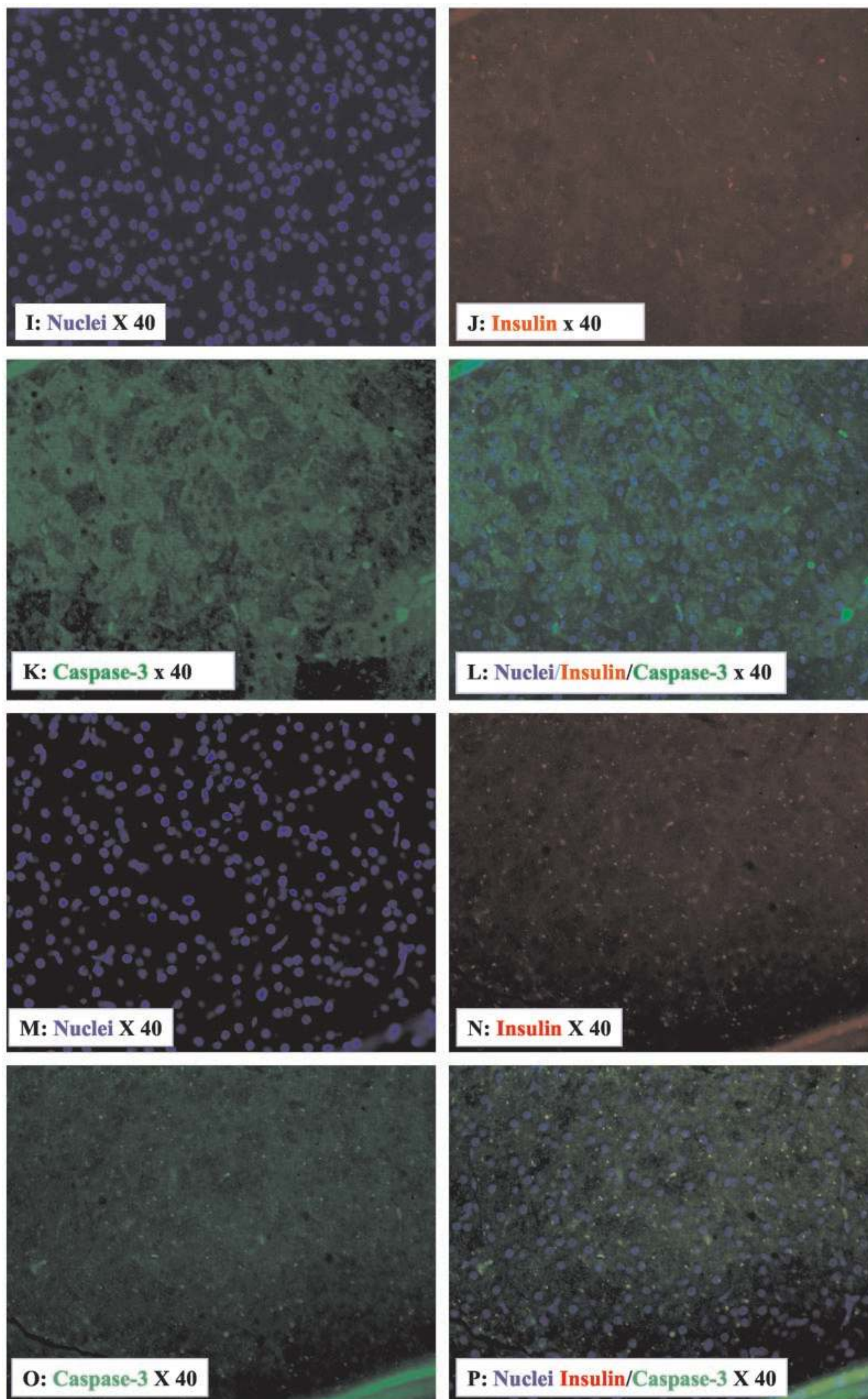
FIG. 6. Immunofluorescence for insulin *(red)* and caspase-3 *(green)* and nuclei *(blue)*. A–D, Caspase-3/insulin immunostaining of islets in saline-infused ZDF rats. A, DAPI nuclear staining; B, insulin; C, caspase-3; D, nuclei, caspase-3, and insulin. E–H, Caspase-3/insulin immunostaining of islets in GLP-1-infused ZDF rats. E, DAPI nuclear staining; F, insulin; G, caspase-3; H, nuclei, caspase-3, and insulin. I–L, Caspase-3/insulin immunostaining of the exocrine pancreas in saline-infused ZDF rats. I, DAPI nuclear staining; J, insulin; K, caspase-3; L,



nuclei, caspase-3, and insulin. M–P, Caspase-3/insulin immunostaining of the exocrine pancreas in GLP-1-infused ZDF rats. M, DAPI nuclear staining; N, insulin; O, caspase-3; P, nuclei, caspase-3, and insulin. All pictures were taken at ×40 magnification, and they were representative of a double staining for insulin and caspase-3 of pancreatic sections obtained from at least five animals per treatment group. At least five sections per each individual pancreas were studied.

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tissue, the exocrine pancreas also showed a healthier status, with a marked down-regulation of the caspase-3 expression in GLP-1-treated animals (Fig. 6, C and D).

The DAPI nuclear staining further confirmed the observation derived from the TUNEL assay and caspase-3/insulin staining, demonstrating a marked decrease of DNA fragmentation in the pancreatic sections obtained from GLP-1-treated rats (Fig. 7).

Quantitative analysis of β-cell mass, β-cell proliferation, and β-cell apoptosis

Treatment of ZDF rats with GLP-1 produced a significant remodeling of islet mass, β-cell replication, and β-cell apoptosis (Fig. 8). ZDF rats treated with GLP-1 showed a 1.6-fold expansion of islet β-cell mass (7.2 ± 1.5 mg in saline-infused rats *vs.* 11.6 ± 1.1 mg in GLP-1-treated rats; $P < 0.01$). This was associated with a 1.4-fold increase in the number of actively dividing β-cells and with an even greater effect of GLP-1 on the number of apoptotic β-cells. Indeed GLP-1 promoted a 3.6-fold decrease in the number of apoptotic β-cells (Fig. 8; $P < 0.001$).

Discussion

In subpancreatectomized diabetic rats (11) and aging glucose-intolerant rats (18), GLP-1 has been shown to be capable of inducing a significant improvement in glucose tolerance. These changes resulted primarily from the proliferation and differentiation of cells of the ductal epithelium into insulin-secreting cells. Interestingly, glucose-dependent insulinotropic polypeptide, an incretin hormone that with GLP-1 regulates postprandial glucose-dependent insulin secretion, has also been shown in a recent report to be capable of promoting the proliferation of the islet-derived β-cell line (19).

By studying the islets of Langerhans of animal models of type 2 diabetes, it has been shown that, although in some of them, the clinical onset of diabetes is the consequence of an impaired proliferative activity of the β-cells; in others, this results from an abnormal rate of cellular apoptosis. The ZDF rat has been shown to develop diabetes when the rate of cell proliferation, which is markedly enhanced, compared with lean controls, no longer compensates for the rate of cell death. GLP-1 has been shown to be capable of improving glucose tolerance by increasing islet cell mass, a mechanism that has been shown to be already activated in ZDF rats and appears to be insufficient in avoiding the onset of diabetes in this animal model. Although these considerations would lead to predict that GLP-1 might not be efficacious in ZDF, clinical investigations have demonstrated a significant improvement of glucose tolerance after treating ZDF rats with GLP-1. The present study was undertaken to characterize the mechanism(s) of action of GLP-1 in ZDF rats and led to demonstrate that GLP-1 had an important effect on β-cell mass, β-cell proliferation, and β-cell mass.

In this study, we investigated the role of GLP-1 on cell proliferation by studying the expression of the Ki-67 antigen, a nuclear protein expressed by dividing cells. Cell cycle analysis has shown that this protein is exclusively expressed during the cell division cycle in the late G1, S, and G2 phases as well as in mitosis, and it is absent in the G0 phase and early G1 phases. By immunofluorescence, we observed that the Ki-67 antigen was expressed both in controls and GLP-1-treated rats. However, in GLP-1-treated rats, the signal for Ki-67 was stronger in intensity and more widely distributed throughout the pancreatic parenchyma than in controls. Moreover, GLP-1-treated animals, but never saline-infused animals, presented Ki-67-positive cells as aggregates of cells,

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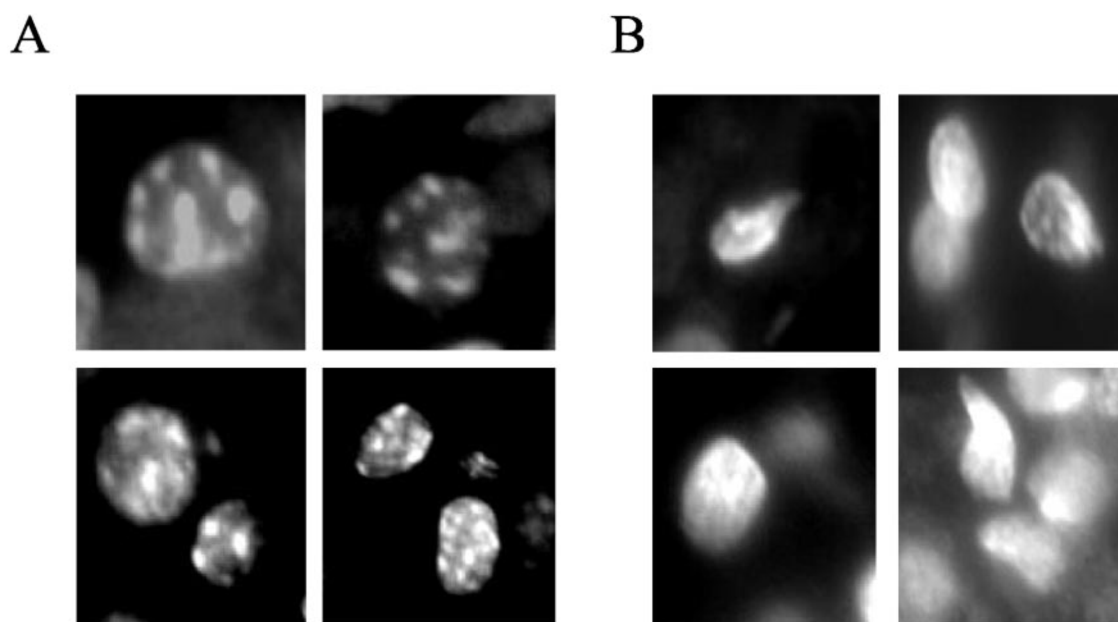

FIG. 7. DAPI staining of nuclei. Details of nuclei of islet cells from saline-infused (A) and GLP-1-treated ZDF rats (B). Pictures are representative of nuclear staining of pancreatic sections of at least five animals per treatment group. At least five sections per each individual pancreas were studied. Pictures of islet cell nuclei are presented at the final magnification of ×100 obtained by microscope magnification and computer-generated enlargement.

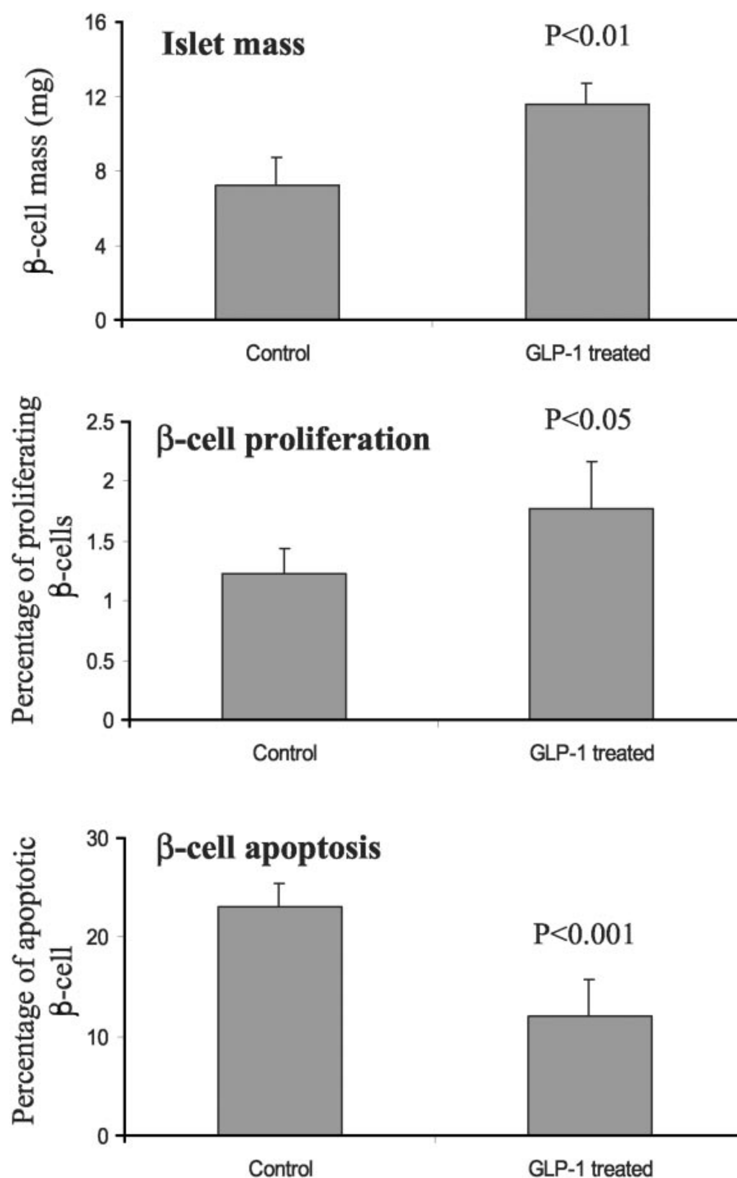

FIG. 8. Quantitative analysis of β-cell mass, β-cell proliferation, and β-cell apoptosis. Four 12-wk-old ZDF rats per treatment group were used for point-counting morphometry to determine islet β-cell mass (A), β-cell proliferation (B), and β-cell apoptosis (C). All sections were stained for insulin; some sections were also costained for Ki-67 to assess the percentage of proliferating β-cells; others were subjected to double staining with the nuclear dye DAPI, together with an antiinsulin antibody to determine the rate of β-cell apoptosis. A total of approximately 200 fields of pancreatic sections was acquired for morphometric analysis. Statistical evaluation of the data was performed by unpaired *t* test.

detected both within and outside the islets of Langerhans. When present among cells constituting the islets, those clusters of proliferating cells never costained for insulin. This could be explained by hypothesizing that GLP-1 is capable of switching on genes that are involved not only in β-cell differentiation but also cell proliferation. Characteristically, in GLP-1-treated rats, the insulin-positive cells appeared to surround aggregates of Ki-67-positive cells. This was different from the appearance of the rare Ki-67-positive cells that were detected in control, saline-infused rats, in which they appeared only as isolated single cells scattered throughout the islets. The detection of cells that were Ki-67 positive but did contain detectable insulin could be attributed to the presence of cells that were actively dividing at the time of tissue harvesting but not yet differentiated. The characterization of these cells will need further studies.

The expression of insulin was higher in GLP-1-treated rats, and the size of the islets in this treatment group was consistently greater than in controls. GLP-1-treated rats had islets that were more irregular in shape as well as a greater number of small aggregates of few insulin-positive cells immersed within the exocrine parenchyma. This irregularity of the margins of the islets could be seen also in controls; however, it appeared significantly more represented after treatment with GLP-1.

An additional set of insulin-positive cells in GLP-1-treated ZDF rats was represented by cells that grew as a mono- or bilayer of cells surrounding small-sized ducts. Those aggregates of insulin-positive cells did not appear to be proximal to gain the appearance of islets, and they often tended to form a net of cells that extended around two or more small-sized ductal structures. Those layers of insulin-positive cells, displayed as a mantel around ducts, were only rarely observed in control rats.

Apoptosis, or programmed cell death, is a physiological mode of remodeling tissue during organogenesis and adulthood. Several studies have demonstrated that the pancreas is actively remodeled after birth and that cell apoptosis is an important mechanism in this process (20). Apoptotic cell death is an energy-requiring process that involves *de novo*

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synthesis of proteins. The process is characterized by morphological changes, including condensation of the nuclear chromatin, DNA fragmentation, cellular shrinkage, and the formation of apoptotic bodies, which are membrane-bound cellular constituents (21). Bonner-Weir and colleagues (14) have shown that the ZDF rat is a model for the failure of β-cell compensation in the presence of an increase of insulin demand because of a preexisting insulin resistance state. This failure is caused by an excess of cell apoptosis in a setting in which cell replication, with age, becomes unable to compensate for the rate of cell loss. In ZDF rats, β-cell mass and β-cell replication rate are greater than in a partially outbred strain that develops obesity and insulin resistance but not diabetes (12, 13). Indeed, in ZDF rats, the onset of diabetes is caused by an excessive rate of β-cell death, not by an inefficient replication capacity (14, 22).

In the present study, we confirmed the previously reported observation describing the presence of a very high percentage of apoptotic nuclei in the pancreas of ZDF rats (14). 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 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.

Quantitative analysis of β-cell mass, β-cell replication, and β-cell apoptosis demonstrated the presence of heterogeneous and cooperative mechanisms by which ZDF rats improve their β-cell function in response to GLP-1. Indeed, GLP-1 not only induced an increase in β-cell replication, as suggested by previously reported findings in other animal models of diabetes and hyperglycemia (11, 18), but it was also responsible for a significant (a perhaps even more important) inhibition of β-cell apoptosis. The latter is a newly identified biological property of GLP-1. Although in assessing mass and proliferation of insulin-producing cells, we applied a methodology extensively described by Pick *et al.* (14) and by Weibel (16), to assess the percentage of apoptotic β-cells, we employed a minor modification of this technique. The number of apoptotic β-cells was determined by counting the number of insulin-positive cells that showed the presence of fragmented nuclei. Although this approach may provide a good specificity assay to measure β-cell apoptosis, it may be defective in sensitivity, providing a low estimate of the number of apoptotic cells. Indeed, the detection of cells with fragmented nuclei that still have a preserved cytoplasmic structure (*i.e.* still susceptible to be stained for the presence of insulin) may lead to miss that population of cells in which the regulation of factors leading to cell apoptosis has already began, but the morphological changes of cells nuclei are not yet detectable.

In conclusion, we provide evidence that GLP-1 ameliorates the glucose tolerance of ZDF rats by inhibiting cell apoptosis and promoting islet-cell proliferation. This finding may further support the clinical investigation for the use of GLP-1 in the treatment of diabetes in humans.

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