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Glucagon-Like Peptide-1 Induces Cell Proliferation and Pancreatic-Duodenum Homeobox-1 Expression and Increases Endocrine Cell Mass in the Pancreas of Old, Glucose-Intolerant Rats

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ABSTRACT

Glucose homeostasis in mammals is maintained by insulin secretion from the β-cells of the islets of Langerhans. Type 2 diabetes results either from primary β-cell failure alone and/or a failure to secrete enough insulin to overcome insulin resistance. Here, we show that continuous infusion of glucagon-like peptide-1 (7–36) (GLP-1; an insulinotropic agent), to young and old animals, had effects on the β-cell of the pancreas other than simply on the insulin secretory apparatus. Our previous studies on a rodent model of glucose intolerance, the aging Wistar rat, show that a plateau in islet size, insulin content, and β-cell mass is reached at 13 months, despite a continuing increase in body weight. Continuous sc infusion of GLP-1 (1.5 pM/kg·min), over 5 days, resulted in normal glucose tolerance. Our current results in both young and old rats demonstrate that treatment caused an up-regulation of pancreatic-duodenum homeobox-1 (PDX-1) expression in islets and total pancreas, induced pancreatic cell proliferation, and β-cell neogenesis. The effects on levels of PDX-1 messenger RNA were abrogated by simultaneous infusion of Exendin (9–39), a specific antagonist of GLP-1. PDX-1 protein levels increased 4-fold in whole pancreata and 6-fold in islets in response to treatment. β-cell mass increased to 7.2 ± 0.58 from 4.88 ± 0.38 mg, treated *vs.* control, respectively, $P < 0.02$. Total pancreatic insulin content also increased from 0.55 ± 0.02 to 1.32 ± 0.11 μg/mg total pancreatic protein. Therefore, GLP-1 would seem to be a unique therapy that can stimulate pancreatic cell proliferation and β-cell differentiation in the pancreas of rodents. (*Endocrinology* **141:** 4600–4605, 2000)

WE HAVE SHOWN previously that, as Wistar rats age, they demonstrate a progressive impairment in glucose-mediated insulin secretion, a reduction in pancreatic insulin content, and insulin messenger RNA (mRNA) synthesis. We also observed a marked reduction in the mRNA levels for the β-cell-specific glucose transporter, GLUT2 (1). This age-dependent decline in β-cell function is also observed in islets isolated from the old rats, demonstrating that abnormalities in insulin secretion occur independently of the declining insulin sensitivity in the aging animal as a whole. After 13 months, we begin to observe a decrease in the number of glucose-responsive β-cells and in the amount of insulin released per cell, despite a progressive increase in body mass (1). The defects attributed to glucose-mediated insulin secretion were reversed by an infusion, *via* microosmotic pump, of glucagon-like peptide-1(7–36) amide (GLP-1), an incretin hormone secreted by the L cells of the intestine (2). The reduction in intraislet insulin content, and insulin and GLUT2 mRNA levels observed with age, were also reversed by treatment with GLP-1 (2).

GLP-1 is a potent insulinotropic hormone (3), which binds to a specific cell surface receptor belonging to the secretin/glucagon superfamily of receptors coupled to heterotrimeric G proteins. The subsequent activation of adenylyl cyclase is believed to mediate the plethora of intracellular effects observed with GLP-1 treatment. The insulinotropic action of GLP-1 is preserved in patients with diabetes, even several years after diagnosis (4). This is similar to the situation in Wistar rats, which are glucose intolerant from 13 months of age. Also consistent with our animal model, when administered by sc injection for 48 h to subjects with type 2 diabetes whose fasting blood glucose was poorly controlled on diet and sulfonylurea therapy, GLP-1 lowered the fasting and postprandial glucose levels (5).

Recently, we demonstrated, in an insulinoma cell line, that GLP-1 enhanced the expression of the transcription factor pancreatic-duodenum homeobox-1 (PDX-1, variously known as IDX-1, STF-1, or IPF-1) (6). PDX-1 expression is essential for pancreogenesis, as demonstrated by the mouse homozygous knockout model and the recent human *pdx-1* inactivating mutation, both of which exhibit pancreatic agenesis (7, 8). In the adult pancreas, PDX-1 regulates genes associated with pancreatic cell differentiation and maturation. These include the insulin, glucokinase, GLUT2, and amyloid precursor protein genes (9).

In this present report, we expanded on our previous research on Wistar rats by more fully characterizing the mechanism of actions of GLP-1, because GLP-1 and/or its agonists (10) may become accepted treatments for diabetes in humans. An up-regulation of PDX-1 could explain the effects we saw on insulin and GLUT2 gene expression, as well as the increase in intraislet insulin levels. Furthermore, if GLP-1 were recruiting new cells from precursor and/or differenti-

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ated β-cells within the islets this would greatly increase the pool of existing insulin secreting cells and perhaps explain some of the long-term effects observed after exposure to GLP-1 (*i.e.* increase in intraislet insulin content). We considered that cell proliferation and the subsequent induction of insulin gene transcription would result from increased expression of homeobox protein(s) that regulates both islet cell mass and function. For both of these reasons, we investigated the effects of GLP-1-induced PDX-1 expression and new endocrine cell formation in our aging glucose-intolerant animals.

Methods and Methods

Materials

GLP-1 and Exendin(9–39) (Ex), a GLP-1 receptor antagonist, were purchased from Bachem Bioscience Inc. (Torrance, CA). Chemical reagents were from Sigma (St. Louis, MO), unless otherwise stated. Insulin RIA kits were from Peninsula Laboratories, Inc. (Belmont, CA). Polyvinylidene difluoride membranes and SDS-polyacryamide gels were from Novex (San Diego, CA). Enhanced chemiluminescence detection reagent was from Amersham Pharmacia Biotech Inc. (Arlington Heights, IL).

Animals

Twenty-two-month- and 6-month-old Wistar rats from the colony at the National Institute on Aging, Gerontology Research Center (Baltimore, MD) were implanted with Alzet microosmotic pumps (Alza Corp., Palo Alto, CA) in the interscapular region for 2 (for analysis of *pdx-1* and insulin genes) or 5 days (for the ip glucose tolerance test, immunocytochemistry, and protein levels). A half-inch nick was made in the skin after the hair had been shaved. A pocket was then made sc, into which the pump was slid. The skin was closed with wound clips (Clay Adams Brand 9 mm, Sparks, MD). In the treated group, GLP-1 was delivered at the rate of 1.5 pmol/kg·min. This gives a plasma level of GLP-1 of 125 ± 41 pM (normal fasting levels are less than 10 pM) (2). Ex, a competitive antagonist of GLP-1, was infused at the rate of 15 pmol/kg·min in the relevant experiments. To prevent the insulintropic action of GLP-1, a 10-fold higher concentration of Ex is required (11).Three additional control groups were infused with either normal saline, Ex (15 pmol/kg·min), or the noninsulinotropic GLP(1–36) (1.5 pmol/kg·min). All animals were fasted from 2300 h on the night before each experiment.

Glucose tolerance testing

This was completed as outlined previously by us (2). The pumps were removed 2 h before testing.

Pancreatic insulin content

Pancreata were rapidly excised and placed individually in 10 vol of ice-cold 75% ethanol containing 1 M acetic acid and 0.1 M HCl. The tissue was homogenized with a Brinkmann/KINEMATICA, Polytron PT3100 (Westbury, NY) using a setting of 7 for 15 sec × 3. Insulin content was subsequently determined as before (1). An aliquot of the above homogenate was centrifuged at 2,000 × *g* for 5 min. The pellet and supernatant were lyophilized and resolubilized in formic acid. The protein content was determined by the Bradford method, and the amount of protein measured in both supernatant and pellet were combined and used as the corrective factor for determination of insulin levels (1).

Isolation of islets

Islets of Langerhans were isolated as described previously (1, 2). Briefly, the whole pancreas was perfused with HBSS containing 1.1% collagenase V, dissected into pieces of approximately 1 mm, and further digested with 0.75% collagenase V for 25 min. Islets were hand-picked and used for Western blot analysis of PDX-1 and for Northern blot analysis.

RNA extraction and Northern blot analysis

Northern blot analysis of total RNA extracted from isolated islets and whole pancreata was performed as previously described (1). RNA was transferred to nylon membranes, hybridized with ^{32}P-labeled insulin (a gift from Dr. S. J. Giddings, Washington University, St. Louis, MO), PDX-1 (a gift from Dr. C. V. E. Wright, Vanderbilt University Medical School, Nashville, TN), or β-actin complementary DNA probes. Membranes were hybridized and washed as described previously (1). Messenger RNA levels were quantified by densitometric analysis by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA) and normalized for β-actin.

Protein extraction for Western blotting from whole pancreas and islets

Whole-cell protein extract was performed according to a modification of the method of Schreiber *et al.* (12). Fasted rats were killed; pancreata were removed and weighed. The following process was performed at 4 C; all solutions, tubes, and centrifuges were chilled to 4 C. Phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were added to the buffers just before use. Minced tissue was brought to a final vol of 30 ml with homogenization buffer containing 20 mM HEPES (pH 7.6), 25 mM KCL, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM Na_3VO_4, and 10% glycerol and was homogenized using a motor-driven Teflon-glass homogenizer until more than 90% of the cells were lysed. The homogenized tissue solution was centrifuged at 1,500 × *g* (3,000 rpm) for 5 min and washed with 10 ml cold PBS containing 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF,10 μg/ml aprotinin, and 20 μg/ml leupeptin, at 1500 × *g* for 5 min. The pellet was resuspended in 100 μl of a cold hypertonic buffer supplemented with 20 mM HEPES (pH7.9), 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 0.125 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.42 M NaCl, 20% glycerol, and 0.1%Triton X-100. The samples were snap-frozen on dry ice and later thawed on wet ice for analysis. The tubes were vigorously vortexed for 10 sec and rocked at 40 C for 30 min, to destroy any remaining whole cells, and centrifuged at 16,000 × *g* (14,000 rpm) for 20 min. Then the supernatant was removed. Aliquots of the supernatant were used to quantify protein content by the Bradford method. Protein (20 μg) was separated on SDS 4–12%-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and probed with the anti-PDX-1 antibody (1:5000; a gift from Dr. Joel Habener, Massachusetts General Hospital, Boston, MA). Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection system.

Islets from 22-month-old animals, treated with or without GLP-1 (six animals per condition), were washed with 1 ml cold PBS containing 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF,10 μg/ml aprotinin ,and 20 μg/ml leupeptin, at 1500 × *g* (3000 rpm) for 5 min. The rest of the procedure is as outlined above. Autoradiographs were quantified using ImageQuant software (version 3.3) on a Molecular Dynamics, Inc. laser densitometer.

Immunohistochemistry

Pancreata were fixed in Bouin's fixative and embedded in paraffin. Five-micron sections were analyzed by an immunohistochemical method with insulin (Linco Research, Inc., St. Charles, MO) and proliferative cell nuclear antigen (PCNA) antisera (Roche Molecular Biochemicals, Indianapolis, IN), stained with 3,3′-diaminobenzidine, and costained with hematoxylin and eosin (13).

β-cell mass and counting of PCNA-positive cells

β-cell mass was determined by point counting in the manner of Pick *et al.* (14) at a final magnification of ×420. Measurements were performed on an Optiphot-2 microscope (Nikon, Melville, NY) and the images were collected on a Sony CCD camera (Dage MTI, Michigan City, IN) and projected to a Sony color monitor with a 96-point transparent (Arkright transparency film, Johnson, RI) overlay used for point count-

ing. The number of points over β-cells was counted, β-cells being those staining with the insulin antiserum. Approximately 200 fields per pancreas were acquired. The percentage of PCNA-positive cells (cells with brown nuclei) was determined by systematically scanning each slide at a final magnification of ×400. Every cell on a slide was also counted. The number of positive nuclei was expressed as a percentage of the total number scanned.

Statistical analysis

The data are expressed as mean ± SEM. Data were analyzed using the nonpaired Student's *t* test; $P < 0.05$ was judged significant.

Results

Twenty-two-month-old Wistar rats, subjected to a 5-day GLP-1 infusion, exhibited a significant decrease in fasting plasma glucose and an increase in fasting plasma insulin levels. After an ip glucose tolerance test (1 g/kg) the area under the curve (0–15 min) for insulin was increased significantly ($P < 0.05$; Table 1). This was associated with a significant increase in total pancreatic weight ($P < 0.02$) and insulin content ($P < 0.001$) (Table 1).

GLP-1 up-regulates pdx-1 mRNA in islets

Northern blot analysis of total RNA extracted from islets of untreated 6- and 22-month-old (n = 6 per age group) rats revealed a decrease in *pdx-1* ($P < 0.05$) and insulin mRNA levels ($P < 0.05$) with age (Fig. 1); levels were 50% lower in the older rats, relative to young untreated controls. Treatment with GLP-1 over 48 h resulted in a statistically significant ($P < 0.05$) increase in *pdx-1* and insulin mRNA levels in both age groups. Despite the lower basal levels seen in the older rats, the increase in mRNA for both insulin and *pdx-1* after treatment was comparable to that seen in the young rats.

GLP-1 up-regulates PDX-1 mRNA in whole pancreatic extracts

Northern blot analysis of total pancreatic RNA extracts from 22-month-old rats demonstrated that GLP-1 treatment for 2 days increased *pdx-1* expression (Fig. 2b, compared with Fig. 2a, where saline alone was given). Concomitant administration of Ex with GLP-1 specifically inhibited the induction of *pdx-1* (Fig. 2c) in the whole pancreas. *pdx-1* was down-regulated by the infusion of Ex (Fig. 2d) and inactive GLP(1–36) (Fig. 2e) alone ($P < 0.05$). A longer GLP-1 infusion (5 days) led to a further increase in *pdx-1* expression (Fig. 2f), suggesting no down-regulation of the biological actions of GLP-1 occurred within this time frame. *pdx-1* expression in the adult rodent is confined to mature terminally differentiated insulin- and somatostatin-producing cells (15).

GLP-1 enhances PDX-1 protein expression in islets and whole pancreas

GLP-1 infusion for 2 days increased PDX-1 protein expression 4-fold in protein extracted from whole pancreas of young and old rats. Figure 3, a and b represents PDX-1 protein from two control pancreata, one young (a) and one old (b). The protein levels were 32% lower in the old animals, compared with young ($P < 0.05$, n = 4).

Islets extracted from the pancreata of 6 old control (Fig 3e) and 6 old treated (Fig 3f) animals showed a 6-fold increase in PDX-1 levels with treatment. The amount of PDX-1 per 20 μg of total protein was 2-fold greater in extracts from islets than in those from the whole pancreas.

GLP-1 induces cell proliferation in the pancreas

Pancreatic sections from the rats infused for 48 h with GLP-1 were immunostained for PCNA. PCNA is identical to cyclin and to the auxiliary protein of DNA polymerase-δ and is marker of proliferative activity. Numerous positive nuclei were observed in the ductal epithelium wall as well as in acinar tissue, suggesting that those cells were induced to multiply by progressing from the early to the late G1 phase of the cell cycle (Fig. 4). Very few PCNA-positive nuclei were observed within the islets of Langerhans. Although the labeling index of all three pancreatic components (ductal, acinar, and endocrine) increased, the relative number of ductal cells induced toward cell mitosis was higher than the other pancreatic cell types. Small- to medium-size pancreatic ducts exhibited up to 80% positivity for PCNA. Investigation of staining in the smaller ducts revealed PCNA positivity in the entire ductal epithelium wall, and the presence of mitotic figures was noted. Large pancreatic ducts were also responsive to the GLP-1-dependent proliferative stimulus: an average of 4–6 cells in large ducts were PCNA-positive (Fig. 4d). Numerous PCNA-positive nuclei were also detected within acinar tissue. PCNA was positive in 16.6 ± 3.1% acinar cells from GLP-1-treated animals *vs.* 6.2 ± 1.3% acinar cells from control animals($P < 0.01$). Although isolated PCNA-positive acinar cells were present in all sections analyzed, more frequently these were present in aggregates of a few cells. They were commonly, but not exclusively, localized in close proximity to small- to medium-size ducts. In some cases a bridge of extracellular matrix and a few sparse fibroblast-like cells physically connected PCNA-positive cell aggregates with a nearby pancreatic duct. Finally, the occasional PCNA-stained cell was observed in the main ducts and in scattered cells within connective tissue. The extent of

TABLE 1. Glucose and insulin levels and pancreatic weights in rats treated with GLP-1 or saline for 5 days

	FPG mM	FP Ins. μU/ml	Pan. Wt (g)	Total pan. Ins. μg/mg total pan.pro.	AUC 0–15 min pM. Ins. IPGTT
GLP-1	5.0[a] ± 0.1	61[b] ± 6	1.29[b] ± 0.06	1.32[a] ± 0.11	8466[c] ± 1558
Saline	5.7 ± 0.1	40 ± 3	1.05 ± 0.05	0.55 ± 0.02	3967 ± 1388

$N = 6$. IPGTT, intraperitoneal glucose tolerance test (1 g/kg); FPG, fasting plasma glucose; FP Ins, fasting plasma insulin; pan, pancreas; pro, protein; AUC, area under the curve.

[a] $P < 0.001$, Students' *t* test.

[b] $P < 0.02$.

[c] $P < 0.05$.

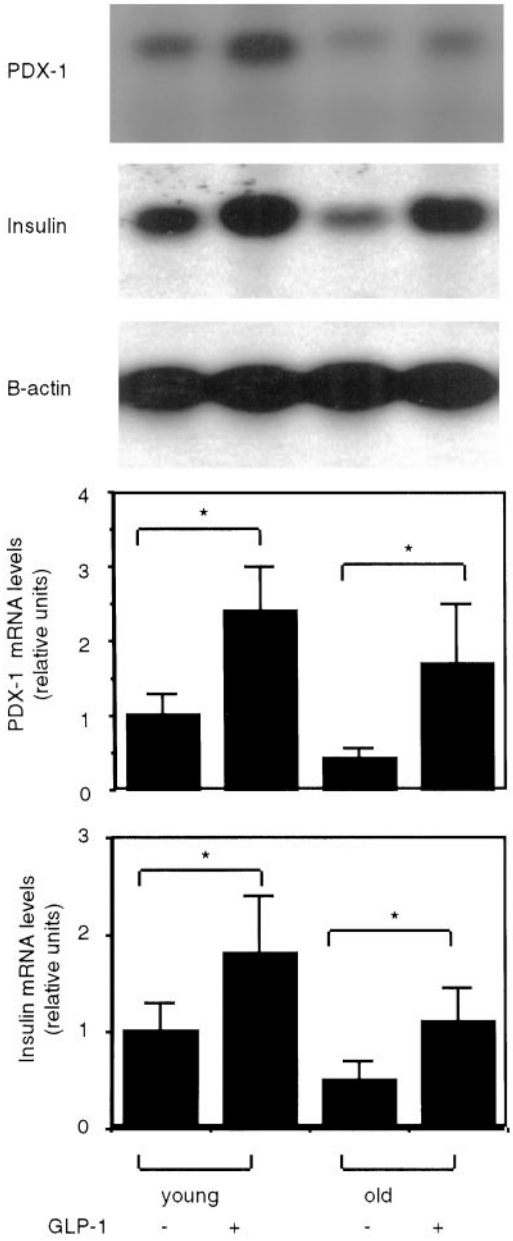

FIG. 1. *pdx-1* and insulin mRNA (10 μg per lane) levels in islets from 6- and 22-month-old rats after a 48-h infusion of GLP-1 or saline. The blot was first hybridized with a rat *pdx-1* complementary DNA probe, followed by the insulin probe. β-actin probe was used to assess RNA loading. The histogram values shown indicate the mean of three independent experiments. Statistical significance was evaluated between islets obtained from GLP-1-treated and nontreated rats, as well as from young *vs.* old rats, before or after treatment. The *P* values shown in the figure were obtained by unpaired Student's *t* test; n = 3 independent experiments; *, $P < 0.05$.

PCNA staining had decreased after 3 days and was no longer present by the fifth day of treatment.

GLP-1 increases β-cell mass

In the saline-treated animals, discrete islets can be seen, with no insulin staining elsewhere (Fig. 5a). In contrast, sections from GLP-1-treated animals exhibit insulin staining in

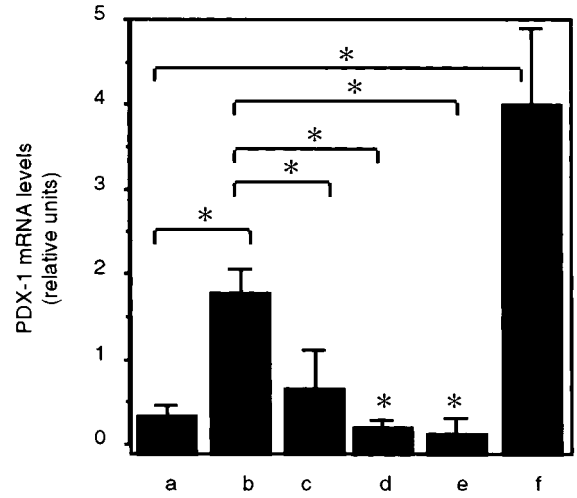

FIG. 2. pdx-1 mRNA levels in total pancreas. RNA was extracted from total pancreas. Whole pancreata of 22-month-old rats infused with normal saline (a), GLP-1(7–36) amide [2-day (b) and 5-day (f)], GLP-1 + Ex [2-day (c)], Ex [2-day (d)], and GLP-1(1–36) amide [2-day (e)] were subjected to RNA extraction and Northern blot analysis (10 μg per lane). Statistical significance was evaluated between pancreata by comparing each individual group with saline-treated, age-matched rats. The *P* values shown in the figure were obtained by unpaired Student's *t* test; n = 3 independent experiments; *, $P < 0.05$. d and e are also significantly different from a ($P < 0.05$).

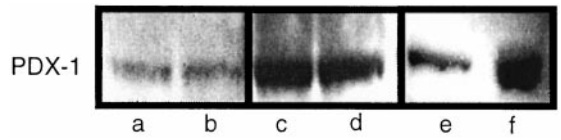

FIG. 3. Western blot analysis for PDX-1 protein levels, 20 μg per lane, from whole pancreata (a–d) as well as islets (e and f) from animals with or without GLP-1 (1.5 pM/kg·min for 2 days). a (young animal), b (old animal) = control pancreata; c (young animal), d (old animal) = pancreata from treated animals. e = control islets, and f = islets from treated old animals (islets were pooled from six animals in each group).

small clusters and ducts as well as in the mature islets (Fig. 5, b–e). These insulin-positive cell clusters were obvious by 5 days (Fig. 5, b and c), but occasional insulin-positive cells could be seen in ducts as early as 3 days. Within the exocrine parenchyma, a few cell aggregates containing insulin also appear. The number of insulin-positive cells present in the epithelial wall of pancreatic ducts was inversely related to the diameter of the duct itself. In the very small ducts, such as in Fig. 5e, most of the cells were insulin-positive. Insulin-positive one- or two-cell pairs were also seen in large-sized ducts (Fig. 5, b and d). Very frequently in the treated animals, individual or paired insulin-positive cells were seen in the acinar regions (Fig. 5, b and c). The mass of the β-cells was also significantly increased by GLP-1 infusion (7.2 $\pm$ 0.58 *vs.* 4.88 $\pm$ 0.38 mg, treated *vs.* control, respectively, $P = 0.02$).

Discussion

The possibility of using GLP-1 as a treatment for type 2 diabetes arose directly from the observation of its insulinotropic activity. The data presented here outline additional biological effects associated with pancreatic cell proliferation

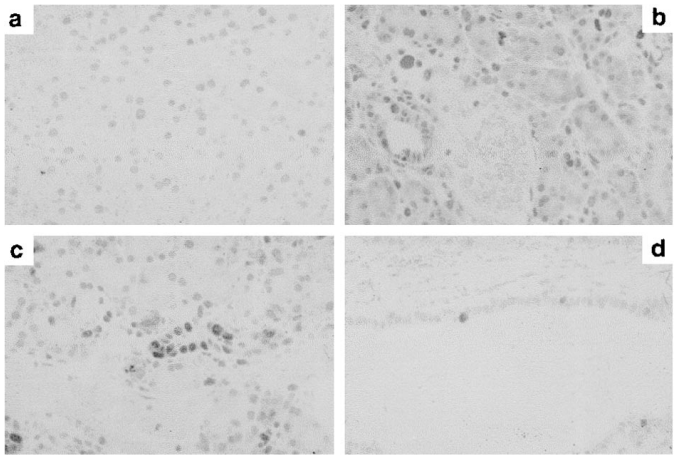

FIG. 4. Immunostaining of pancreatic sections for PCNA in 22-month-old Wistar rats. Twenty-two-month-old Wistar rats underwent GLP-1 infusion (1.5 pM/kg·min) for 2 days using a microosmotic pump, whereas control age-matched rats were infused with saline alone. Animals that had been fasted overnight were killed and their pancreata were imbedded in paraffin and cut into several 5-micron sections. a, Saline-treated; b–d, GLP-1-treated. The cells of some small ducts were sometimes all positive (b and c), with even large ducts having between 4–8 cells positive (d); magnification, 80×.

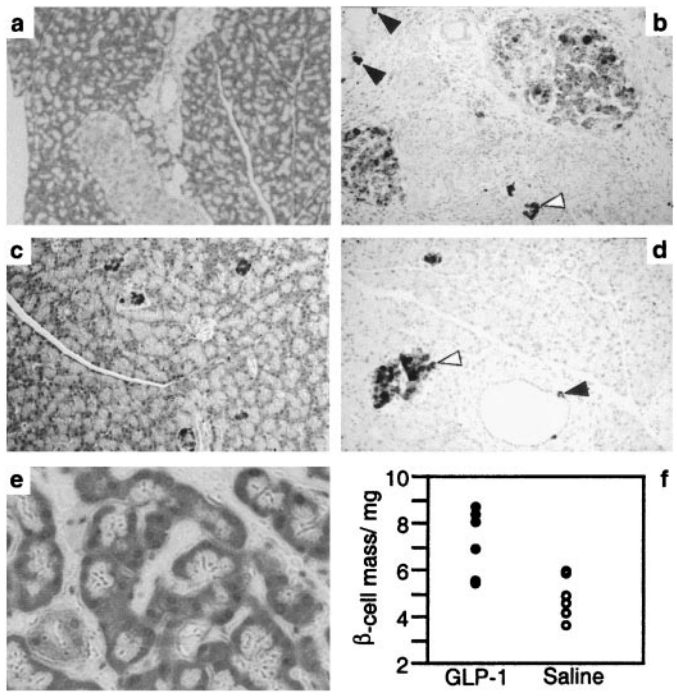

FIG. 5. Immunostaining for insulin and analysis of β-cell mass. Immunostaining for insulin in the pancreas of a 22-month-old rat treated with saline for 5 days (a) or with GLP-1 (1.5 pM/kg·min) continuously for 5 days *via* a microosmotic pump (b–e). f, Quantification of β-cell mass was performed by point-counting analysis on sections immunostained for insulin. The number of insulin-positive cells present in the epithelial wall of pancreatic ducts was inversely correlated with the diameter of the duct itself. In the very small ducts, such as in e, most of the cells were insulin-positive. The *closed arrowhead* points to a few cells positive for insulin even in large ducts. *Open arrowheads* demonstrate aggregates of insulin positivity, b and d, while c shows aggregates only. a–d, Magnification 50×; e, magnification 400×.

and differentiation that are potentially beneficial in the context of long-term treatment, should similar effects occur in human pancreata. Our results indicate that a continuous infusion of GLP-1, over 2–5 days, induces *pdx-1* mRNA and protein expression and islet-like neogenesis. Our data are in agreement with two other reports: one in an insulinoma cell line, INS-1 (16), and another in a rat model of diabetes (17). In the cell line, GLP-1 induced proliferation and up-regulated PDX-1 activity. Exendin-4, a more potent and long-acting agonist of GLP-1 (10), stimulated β-cell replication and neogenesis, causing a 40% increase in β-cell mass. Here, we show that GLP-1 not only increases β-cell mass but is also a proliferative factor in the acini and ducts of the pancreas.

PDX-1 is important for pancreatic development because *pdx-1* nullizygosity results in failure of the pancreas to develop in both mice and a human (7, 8). There is recent evidence of an association between missense mutations of *pdx-1* and type 2 diabetes (18, 19). It is known that β-cell function continues to deteriorate with age in this condition (20). Although it has not been investigated, it may be that β-cell mass and PDX-1 expression are reduced in type 2 diabetes, analogous to what is seen here in the glucose-intolerant aging rat. It is feasible that chronic GLP-1 treatment may retard, or even prevent, this deterioration of the β-cell.

PDX-1 serves a second function in adult life as it regulates genes that are specific to the β-cell. Specifically, it transactivates the promoter regions of the insulin, glucokinase, GLUT2, and amyloid precursor protein genes (9, 21). We have previously demonstrated that GLP-1 infusion, *in vivo*, increases not only insulin gene expression but also glucokinase and GLUT2 expression in young and old animals. These facts, together with the data here, allow us to form an outline of the pathway whereby GLP-1 causes an up-regulation of insulin mRNA and protein. GLP-1 up-regulates PDX-1 expression, which in turn, up-regulates insulin, GLUT2, and glucokinase genes. The macroscopic manifestation of these events is seen in the improved glucose tolerance. The evidence suggests this effect is directly mediated through the GLP-1 receptor, because there is no increase in PDX-1 or insulin expression when GLP-1 is infused in the presence of the antagonist Ex (present data and Ref. 2). Treatment with the inactive insulinotropic form of GLP-1, GLP-1(1–36), did not replicate the effects of GLP-1. There is a decrease in mRNA levels of PDX-1, relative to saline-treated controls, when both Ex and GLP-1(1–36) were administered in isolation. Possible explanations are that Ex is inhibiting the action of endogenous GLP-1 and that the inactive GLP-1(1–36) is abrogating the synthesis of GLP-1.

GLP-1 caused an increase in the proliferative capacity of acinar and duct cells. Our hypothesis is that *pdx-1* expression is subsequently induced with consequent differentiation of some of these proliferating cells to endocrine cells. This is substantiated by the finding that PDX-1 protein is increased not only in islets but in the whole pancreas (this paper and Ref. 22). If PDX-1 were increased in islets alone (and because islets compose only 0.5–1% of the total pancreatic mass), we would be unlikely to appreciate the increase in protein extracted from the whole pancreas as analyzed by Western blot. On the other hand, the expression of PDX-1 in nonislet cells is unlikely to be, *per se*, sufficient to cause differentiation of

all PDX-1-positive cells into endocrine cells. Other differentiation factors are known to be necessary for the full differentiation of the endocrine pancreas (9). The presence of other differentiation factors and/or perhaps specific topography within the pancreatic parenchyma (*i.e.* proximity with ductal structures and/or mature islets) may allow some PDX-1-positive cells to progress toward a fully differentiated β-cell phenotype This may explain the discrepancy reported in the present study between GLP-1-dependent increase in PDX-1 and insulin transcripts. PDX-1 is necessary for the process that leads to the differentiation into β-cell, whereas insulin testifies that the process has reached full completion.

The capacity of GLP-1 treatment to induce PDX-1 and β-cell neogenesis in old animals is of importance in the context of type 2 diabetes. In this report and our previous one (2), the improvement in glucose tolerance can, at least in part, be ascribed to an increase in β-cell mass. At the point we carried out the glucose tolerance testing, the source of exogenous GLP-1 was removed 2 h previously; thus, GLP-1 levels in plasma were below the detection limit (2). Improved glucose tolerance cannot, therefore, be ascribed to a direct incretin effect. As type 2 diabetes occurs in the older age group, this is the population that is likely to receive a GLP-1 type compound in the future. Our data, therefore, are pertinent to the clinical application of GLP-1.

References

1. **Perfetti R, Montrose-Rafizadeh C, Liotta A, Egan JM** 1995 Age-dependent reduction in insulin secretion and insulin mRNA in isolated islets from rats. Am J Physiol 269:E983–E990
2. **Wang Y, Perfetti R, Greig N, Holloway H, DeOre K, Montrose-Rafizadeh C, Elahi D, Egan JM** 1997 Glucagon-like peptide-1 can reverse the age-related decline in glucose tolerance in rats. J Clin Invest 99:2883–2889
3. **Thorens B, Waeber G** 1993 Glucagon-like peptide-1 and the control of insulin secretion in the normal state and in NIDDM. Diabetes 42:1219–1225
4. **Nauck M, Kleine AN, Orskov C, Holst JJ, Willms B, Creutzfeldt W** 1993 Normalization of fasting hyperglycemia by exogenous glucagon-like peptide 1 (7–36) amide in type 2 (non-insulin-dependent) diabetic patients. Diabetologia 36:741–744
5. **Toft-Nielsen M-T, Madsbad S, Holst JJ** 1999 Continuous subcutaneous infusion of glucagon-like peptide lowers plasma glucose and reduces appetite ‘in type 2 diabetic subjects. Diabetes Care 22:1137–1143
6. **Wang X, Cahill CM, Pineyro MA, Zhou J, Doyle ME, Egan JM** 1999 Glucagon-like peptide-1 regulates the beta cell transcription factor, PDX-1, in insulinoma cells. Endocrinology 140:4904–4908
7. **Jonsson J, Carlsson L, Edlund T, Edlund H** 1994 Insulin-promoter-factor-1 is required for pancreas development in mice. Nature 371:606–609
8. **Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener JF** 1997 Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet 15:106–110
9. **Habener JF, Stoffers DA** 1998 A newly discovered role of transcription factors involved in pancreatic development and the pathogenesis of type 2 diabetes mellitus. Proc Assoc Am Physician 110:12–21
10. **Greig N, Halloway HW, De Ore K, Wang A, Jani YD, Zhou J,Garant M, Egan JM** 1999 Once daily injections of Exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose levels. Diabetologia 42:45–50
11. **Wang Z, Wang RM, Owji AA, Smith DM, Ghatei MA, Bloom SR** 1995 Glucagon-like peptide-1 is a physiological incretin in man. J Clin Invest 95:417–421
12. **Schreider E, Matthias P, Muller MM, Schaffer W** 1989 Rapid detection of octamer binding proteins with “mini-extracts”, prepared from a small number of cells. Nucleic Acids Res 17:6419–6422
13. **Zhou J, Wang X, Pineyro MA, Egan JM** 1999 GLP-1 and Exendin-4 convert AR42J cells into insulin- and glucagon-containing cells. Diabetes 48:2358–2366
14. **Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS** 1998 Role of apoptosis in failure of β-cell mass compensation for insulin resistance and β-cell defects in the male Zucker diabetic fatty rat. Diabetes 47:358–364
15. **Herrera PD** 2000 Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127:2317–2322
16. **Buteau J, Roduit R, Susini S, Prentki M** 1999 Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. Diabetologia 42:856–864
17. **Xu G, Stoffers DA, Habener JF, Bonner-Weir S** 1999 Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276
18. **Macfarlane WM, Frayling TM, Ellard S, Evans JC, Allen LIS, Bulman MP, Ayres S, Shepherd M, Clark P, Millward A, Demaine A, Wilkin T, Doherty K, Hattersley AT** 1999 Missense mutations in the insulin promoter factor-1 gene predispose to type 2 diabetes. J Clin Invest 104:R33–R39
19. **Hani EH, Stoffers DA, Chevre JC, Durand E, Stanojevic V, Dina CD, Habener JF, Froguel P** 1999 Defective mutations in the insulin promoter factor-1 (IPF-1) gene in late-onset type 2 diabetes mellitus. J Clin Invest 104:R41–R48
20. **Cook JTE, Page RCL, Hammersley MS, Walvarens EKN, Turner RC** 1993 Hyperglycemic progression in subjects with impaired glucose tolerance: association with decline in beta cell function. Diabet Med 10:321–326
21. **Waeber G, Thompson N, Nicod P, Bonny C** 1996 Transcriptional activation of the GLUT2 gene by IPF-1/STF-1/IDX-1 homeobox domain. Mol Endocrinol 10:1327–1334
22. **Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, Egan JM** 2000 Insulinotropic glucagon-like peptide-1 agonists stimulate expression homeoprotein IDX-1 and increase islet mass in mouse pancreas. Diabetes 49:741–748