Dipeptidyl Peptidase IV Inhibitor Treatment Stimulates β-Cell Survival and Islet Neogenesis in Streptozotocin-Induced Diabetic Rats

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Recent studies into the physiology of the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) have added stimulation of β-cell growth, differentiation, and cell survival to well-documented, potent insulinotropic effects. Unfortunately, the therapeutic potential of these hormones is limited by their rapid enzymatic inactivation in vivo by dipeptidyl peptidase IV (DP IV). Inhibition of DP IV, so as to enhance circulating incretin levels, has proved effective in the treatment of type 2 diabetes both in humans and in animal models, stimulating improvements in glucose tolerance, insulin sensitivity, and β-cell function. We hypothesized that enhancement of the cytoprotective and β-cell regenerative effects of GIP and GLP-1 might extend the therapeutic potential of DP IV inhibitors to include type 1 diabetes. For testing this hypothesis, male Wistar rats, exposed to a single dose of streptozotocin (STZ; 50 mg/kg), were treated twice daily with the DP IV inhibitor P32/98 for 7 weeks. Relative to STZ-injected controls, P32/98-treated animals displayed increased weight gain (230%) and nutrient intake, decreased fed blood glucose (~26 vs. ~20 mmol/l, respectively), and a return of plasma insulin values toward normal (0.07 vs. 0.12 nmol/l, respectively). Marked improvements in oral glucose tolerance, suggesting enhanced insulin secretory capacity, were corroborated by pancreas perfusion and insulin content measurements that revealed two- to eightfold increases in both secretory function and insulin content after 7 weeks of treatment. Immunohistochemical analyses of pancreatic sections showed marked increases in the number of small islets (+35%) and total β-cells (+120%) and in the islet β-cell fraction (12% control vs. 24% treated) in the treated animals, suggesting that DP IV inhibitor treatment enhanced islet neogenesis, β-cell survival, and insulin biosynthesis. In vitro studies using a β-(INS-1) cell line showed a dose-dependent prevention of STZ-induced apoptotic cell-death by both GIP and GLP-1, supporting a role for the incretins in eliciting the in vivo results. These novel findings provide evidence to support the potential utility of DP IV inhibitors in the treatment of type 1 and possibly late-stage type 2 diabetes. *Diabetes* 52:741–750, 2003

Despite substantial advances in our understanding of type 1 diabetes, diagnosis of the condition still carries with it a sentence of lifelong daily insulin injection, a partially effective therapy at best (1). New therapeutic strategies under investigation include islet transplantation (and associated stem cell–derived and xenogeneic islet technologies necessary for treatment *en masse*), the development of improved insulin analogues and delivery systems, gene therapy, and the search for novel agents able to protect and/or stimulate the proliferation and regeneration of islet β-cells (1). The importance of the latter strategy is underscored by the need for an inexpensive, benign, preventive therapy that lacks the considerable profile of side effects of most therapies studied to date (e.g., immunosuppressants).

A number of recent studies have highlighted the role of the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) in β-cell function and development (2,3). Together, the incretins are responsible for >50% of nutrient-stimulated insulin release and make up the endocrine arm of the enteroinsular axis (4). Apart from their insulinotropic role, GIP and GLP-1 have been shown to enhance β-cell glucose competence and, more recent, to stimulate β-cell growth, differentiation, proliferation, and cell survival (5–11). Inhibition of dipeptidyl peptidase IV (DP IV), a ubiquitous serine protease that rapidly cleaves and inactivates GIP and GLP-1 in vivo, has been shown to raise circulating active incretin levels and thus increase the effective concentration of these peptides reaching target tissues (12–16). Studies in both humans and animal models have established DP IV inhibition as a promising therapeutic strategy for the treatment of type 2 diabetes (17–23). We recently showed that long-term administration of the DP IV inhibitor P32/98 to VDF rats (model of type 2 diabetes)

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DP IV, dipeptidyl peptidase IV; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; IDGTT, intraduodenal glucose tolerance test; IR, immunoreactive; OGTT, oral glucose tolerance test; PDX-1, pancreatic-duodenum homeobox-1; PP, pancreatic polypeptide; SOM, somatostatin; STZ, streptozotocin.

resulted in an enhancement of glucose tolerance, insulin sensitivity, and β-cell glucose responsiveness (19,23). Considering the pleiotropy of noninsulinotropic incretin effects that culminate in an overall promotion of β-cell function and survival, we hypothesized that DP IV inhibitor treatment of rats transiently exposed to the β-cell–specific toxin streptozotocin (STZ) would be able to protect islet β-cells from the initial toxic insult and possibly to stimulate β-cell proliferation and function.

In the current study, we show that long-term DP IV inhibitor treatment of STZ rats improves glucose tolerance, enhances pancreatic insulin content, and stimulates islet neogenesis and the survival of pancreatic β-cells. Furthermore, using an in vitro β-cell model (INS-1 832/13), we show a dose-dependent prevention of STZ-induced apoptosis by GIP and GLP-1, providing a potential mechanism of action for the in vivo findings. These findings provide a basis on which to extend the therapeutic potential of DP IV inhibitors to include type 1 and very-late-stage type 2 diabetes.

RESEARCH DESIGN AND METHODS

Materials. Unless otherwise stated, all materials were obtained from Sigma Chemical (St. Louis, MO). The specific DP IV inhibitor P32/98 (di-[2S,3S]-2-amino-3-methyl-pentanoic-1,3-thiazolidine fumarate) was synthesized as previously described (24).

Animals. Male Wistar rats (250 g) were randomly assigned to one of four groups: control, STZ control, early treatment, or late treatment ($n = 15$). The two treatment groups were administered P32/98 (10 mg/kg) twice daily by oral gavage starting either 1 week before (early) or 1 week after (late) the STZ injection (day 0). The control groups were administered a 1% cellulose injection vehicle. On day 0, all groups save the controls were administered STZ (50 mg/kg i.v., tail vein) while under halothane anesthesia. The control group was administered citrate buffer alone. The techniques used in this study were in compliance with the guidelines of the Canadian Council on Animal Care and were approved by the UBC Council on Animal Care (Certificate A99-0006).

Acute DP IV inhibition during intraduodenal glucose tolerance tests. A second set of Wistar rats (275 g) received an injection of STZ, as described above, to examine the effects of acute DP IV inhibition in the STZ model. Six days after STZ injection, animals were anesthetized (sodium pentobarbital 50 mg/kg) and subjected to two successive intraduodenal glucose tolerance tests (IDGTT; 1 g/kg), first in the absence of a DP IV inhibitor and second after an intravenous bolus of P32/98 (10 mg/kg). Blood samples, taken at regular intervals from a carotid cannula, were collected directly into tubes containing P32/98 (final concentration 1 mmol/l; to prevent further DP IV degradation of GLP-$1_{7–36}$) and placed immediately on ice. Plasma was separated and stored at −20°C within 1 h of collection. Samples were analyzed for active and total GLP-1 using enzyme-linked immunosorbent assay– and radioimmunoassay–based kits, respectively (Linco Research).

Weekly monitoring. During the long-term study, 500-μl blood samples were collected from each animal into heparinized capillary tubes at 9:00 A.M. on a weekly basis. Samples were then centrifuged (15 min at 12,000 rpm) and stored at −20°C until analysis. Blood glucose values were determined using a SureStep Glucose analyzer (LifeScan Canada, Burnaby, BC, Canada). Plasma fructosamine measurements were performed using a LaRoche Diagnostic Fructosamine kit (LaRoche Diagnostics) as per the manufacturer's instructions. Similarly, plasma insulin levels were determined using a rat-specific sensitive insulin kit (0.02–1.0 ng/ml; Linco Research). DP IV activity levels were measured as previously described (19). Food and water intake was measured by subtraction.

Measurement of oral glucose tolerance. Seven weeks after STZ exposure, the animals underwent an oral glucose tolerance test (OGTT; 1 g/kg) after a 16-h fast. In this case, P32/98 was not administered the morning of the OGTT to ensure complete drug washout (16 h from last drug administration; 1.5 h clearance half-life). Blood glucose and plasma insulin measurements were made as above (250-μl samples).

Measurment of pancreatic function. For assessing the effects of long-term P32/98 treatment on pancreatic function in the STZ model, a low-to-high glucose pancreas perfusion was performed as previously described (25). In brief, the pancreas was isolated through a ventral midline incision, all major vasculature was ligated, and a glucose perfusate was introduced through the celiac artery. Perfusion effluent was collected at 1-min intervals via the portal vein with a perfusion rate of 4 ml/min. Samples were stored at −20°C until analysis for insulin.

Total pancreatic insulin. Total pancreatic insulin content was determined in one-third of the experimental subjects using conventional techniques. After induction of anesthesia with sodium pentobarbital (Somnotol; 50 mg/kg i.p.), the pancreas was excised, blotted dry, and weighed. Pancreata were then homogenized in 5 ml of ice-cold 2 N acetic acid, boiled, and centrifuged (10 min, 15,000 rpm, 4°C). The supernatant was then assayed for insulin content and normalized for protein concentration (BCA; Pierce, Rockford, IL).

Immunohistochemistry. Pancreata removed under anesthesia were immediately placed in fixative (44% formaldehyde, 47% distilled H_2O, 9% glacial acetic acid) for 48 h, after which they were washed and stored in 70% ethanol. To account for variation between pancreatic regions, the pancreata were cut into ~4-mm blocks that were then randomly inserted into a cassette and paraffin-embedded. β-Cell counts were made from all blocks (~10–15) in a cassette, thereby ensuring assessment of a randomized set of cross-sections in all three planes throughout each pancreas. Once embedded, pancreata were sectioned (5 μm), and the sections were mounted onto slides and dried ready for staining. Sections were stained for insulin (Rbt α-insulin H-86; Santa Cruz Biotechnology, Santa Cruz, CA) as per the manufacturer's instructions, and the nuclei were counterstained using DAPI (0.3 μmol/l; Molecular Probes, Eugene, OR) to facilitate quantification of β-cell and islet parameters. Quantification was performed manually, as a high degree of variability was observed in insulin staining within islet sections from animals exposed to STZ. Determinations of insulin immunoreactive (IR) cell number and total cross-sectional islet cell number (nuclear stain) were made in islets from 250 mm^2 of pancreatic tissue section per animal (~100 islets); three to four animals were counted in each experimental group.

Protection of β-(INS-1) cells in vitro. The rat insulinoma cell line INS-1 (832/13) was obtained as a gift from Dr. C.B. Newgard (Duke University, Durham, NC). Cells were cultured in RPMI 1640 supplemented with 10% FBS (Cansera, Rexdale, ON), 11 mmol/l glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES (pH 7.4), 2 mmol/l L-glutamine, 1 mmol/l pyruvate, and 50 μmol/l β-mercaptoethanol. Cells were plated into either 6-well (2×10^6 cells/well; caspase studies) or 96-well (7.5×10^4 cells/well; protection assays) coated tissue-culture plates (Becton Dickinson, Lincoln Park, NJ) 24 h before experimentation. Unless otherwise stated, the glucose concentration during all cell survival assays was 3 mmol/l for the duration of the experiment to minimize complications associated with stimulation of proliferation and glucose-dependent secretory products. Similarly, in all experiments, cells were serum-starved overnight (3 mmol/l glucose RPMI + 0.1% BSA) and subjected to a 30-min STZ exposure the next day. After STZ exposure, medium was replaced and cells were allowed to recover for 24 h. GIP and/or GLP-1 (10^{-7}M) was added 10 min before STZ (2 mmol/l) during the dose-response and caspase-3/8 experiments and 10 min before (Pre), immediately after (Post), or both 10 min before and immediately after (Pre + Post) the STZ exposure for the cell protection assays. Post and Pre + Post groups were therefore exposed to incretins for the duration of the 24-h recovery period. Quantification of live versus dead cell number was made manually using a conventional trypan blue exclusion method. Caspase-3/8 determinations were made with the EnzChek Caspase Assay Kits (Molecular Probes) according to the manufacturer's recommendations.

Data analysis and presentation. Data, presented as mean ± SE, were compared with Prism 3.02 data analysis software (GraphPad Software, La Jolla, CA) using ANOVA followed by a Dunnett's multiple comparison test post hoc ($P < 0.05$).

RESULTS

Acute inhibition of DP IV enhances plasma-active GLP-1 and potentiates insulin secretion. In a separate group of STZ rats, an IDGTT was used to demonstrate a DP IV inhibitor–mediated increase in circulating active GLP-1 levels. A 10 mg/kg dose of P32/98 was sufficient to protect an additional 40% of the total GLP-1 secretion, allowing peak glucose-stimulated active GLP-1 levels to reach 45 ± 7 pmol/l (vs. 14 ± 1 pmol/l for control; Fig. 1). Furthermore, an earlier and more robust plasma insulin peak was evidenced after P32/98 treatment as was a concomitant reduction in blood glucose (Fig. 1).

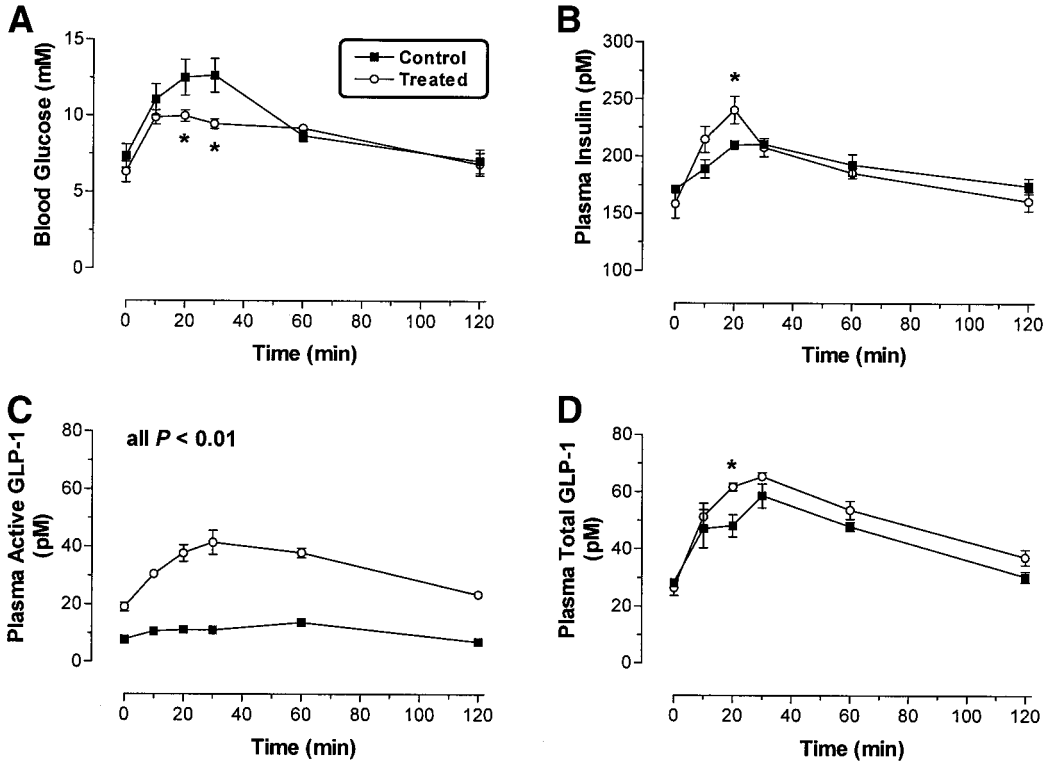

FIG. 1. DP IV inhibition enhances active GLP-1 levels and early-phase insulin release and improves intraduodenal glucose tolerance. Blood glucose (*A*), plasma insulin (*B*), and plasma active (*C*) and total (*D*) GLP-1 levels measured during an IDGTT (1 g/kg) performed in anesthetized STZ Wistar rats either in the presence (open circles) or absence (closed squares) of the DP IV inhibitor P32/98 (10 mg/kg). *Statistical significance ($P < 0.05$) versus control.

Effects of DP IV inhibitor treatment on weight gain and nutrient intake. STZ-induced diabetes in rats is accompanied by a marked reduction in weight gain despite increased caloric intake, a direct result of insulin insufficiency (Fig. 2). Over the course of the present study, the early and late treatment groups gained 129 ± 7 g and 92 ± 4 g, respectively (2.3- and 1.7-fold STZ-control; Fig. 2). Furthermore, the STZ-induced doubling in food intake exhibited by the diabetic controls was decreased 48 and 23% in the early and late groups, respectively. Changes in food and water intake paralleled one another, with improvements evident in both treatment groups within 1 week of treatment initiation (Fig. 2).

Effects of DP IV inhibitor treatment on blood glucose, plasma insulin, and plasma DP IV. The initial STZ-induced increase in morning blood glucose values averaged ~20 mmol/l above normal (Fig. 3*A*). This rapid rise in glycemia, which stabilizes within 3–4 days, was markedly attenuated in the early treatment group plateauing ~5 mmol/l below the untreated controls. In contrast to a gradual deterioration in morning blood glucose in the untreated STZ controls, both treatment groups displayed significant reductions in morning glycemia within 1 week of treatment initiation. The reduction in morning glycemia evident in the late treatment group, unlike the abrupt, immediate reduction seen in the early groups, was progressive during the 6 weeks of treatment. Corroborating the reduced morning glucose values, plasma fructosamine levels in both treatment groups were significantly decreased (Fig. 3*B*). Once again, the early treatment group exhibited an immediate blunting of the STZ-induced rise in plasma fructosamine, whereas the late group showed a more progressive reduction, achieving significance during the final 2 weeks of the study.

Whereas untreated animals displayed a 0.08 nmol/l drop in plasma insulin within the first 2 weeks after STZ exposure, the pretreated animals presented only a 0.04 nmol/l drop, a 43% improvement over the untreated controls. As before, the late treatment group displayed a more gradual reversal, with a 40% elevation in morning plasma insulin levels detected by week 6 of the experiment (Fig. 3*C*). Morning plasma DP IV activity measured immediately before P32/98 administration showed an STZ-induced elevation to a plateau level ~50% above normal within 4 days in all three STZ groups (Fig. 3*D*).

Effects of DP IV inhibitor treatment on oral glucose tolerance in STZ rats. Seven weeks after STZ injection, all groups underwent an OGTT. Fasting blood glucose values in the treated animals averaged 5.4 mmol/l less and 2.0 mmol/l less than their untreated diabetic littermates (early and late, respectively), although neither group achieved normalcy (Fig. 4*A*). Integrated blood glucose responses during the 120-min course of the OGTT were decreased by 33 and 20% in the early and late treatment groups, respectively, with concomitant 240 and 45% increases in integrated insulin responses (Fig. 4, insets). Glucose responsiveness of insulin secretion, which was largely ablated in the diabetic controls, was partially restored in the early treatment group (Fig. 4*B*).

Effects of DP IV inhibitor treatment on pancreatic insulin secretion and content. The partial restoration of glucose responsiveness (of insulin release) observed during the OGTT was accompanied by increased glucose-stimulated insulin secretion during pancreas perfusion.

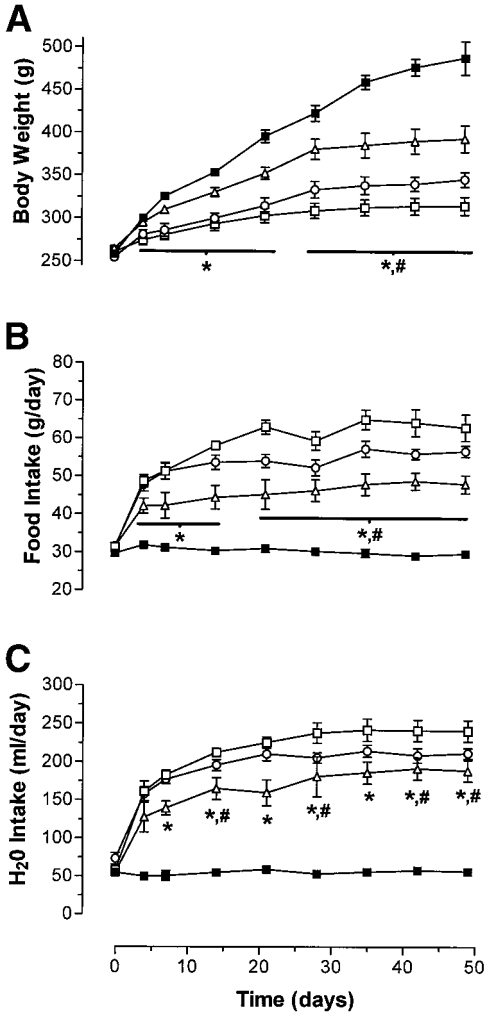

FIG. 2. Body weight (*A*), food intake (*B*), and water intake (*C*) measurements from Wistar rats ($n = 20$) exposed to a single high dose of STZ (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Control (solid squares) and STZ control (open squares) animals were administered a 1% cellulose solution, whereas the early treatment group (open triangles; treatment initiated 1 week before STZ administration) and the late treatment group (open circles; treatment initiated 1 week after STZ administration) were administered 10 mg/kg P32/98 twice daily by oral gavage. Food and water intake was measured by subtraction. *Statistical significance ($P < 0.05$) for the early group versus STZ control; #statistical significance for the late group versus STZ control.

Under basal (4.4 mmol/l glucose) conditions, insulin secretion from pancreata of both treated groups was elevated (190 and 77% for the early and late treatment groups, respectively) compared with the untreated control levels (Fig. 5). Basal secretion from the early treatment group equaled normal Wistar controls (0.21 nmol/l). Furthermore, first-phase insulin release in response to a 4.4–8.8 mmol/l glucose perfusion step was partially restored in both treatment groups with peak insulin levels reaching 0.87 and 0.59 nmol/l in the early and late groups, respectively (~70 and ~50% of control, 400 and 250% of STZ control; Fig. 5*A*).

In keeping with the increases in basal insulin secretion evident during perfusion, whole pancreas insulin determinations revealed parallel increases in insulin content after long-term DP IV inhibitor treatment (Fig. 5*B*). The late and early treatment groups exhibited greater than two- and eightfold higher total pancreatic insulin levels than the untreated STZ-injected group (corresponding to 5 and 17% of control). Data presented in Fig. 5, normalized against protein concentration, are representative of total pancreatic insulin as no difference was evidenced in pancreatic mass between groups (control 1.91 ± 0.10 g; STZ control 1.93 ± 0.11 g; early treatment 1.84 ± 0.07 g; and late treatment 1.86 ± 0.06 g).

Effects of DP IV inhibitor treatment on β-cell number and islet morphology. Pursuant to evidence of increased pancreatic insulin content, morphometric analysis of islet β-cell distribution was performed. Examination of islet number per unit area in all three STZ-exposed groups showed an increase in the number of small islets (1–20 cells in cross-section; Fig. 6*A*). The effect was most profound in the treated groups, with marked increases in islets with a cross-sectional area ≤10 cells. Determination of β-cell area as a fraction of cross-sectional islet cell number revealed that the majority of islets ≤20 cells in cross-sectional area contained a near-normal fraction of β-cells (60–80%), whereas those of larger size showed a marked reduction in β-cell fraction (Fig. 6*B*). Significant increases in β-cell fraction were evidenced in the early treatment group in all subsets of islets >10 cells in cross-section, whereas a significant increase in the late group was seen in only the group 11–20 cells in cross-section (Figs. 6*B* and *D*). The logical interpretation of these two results, in functional terms, is that β-cells in small islets make up a larger fraction of the functional β-cell mass (insulin-IR mass) in the three STZ groups. Expressed as the fraction of the total pancreatic β-cell number, β-cells of smaller islets were shown to compose a significantly larger fraction of the total β-cell mass in all three STZ-exposed groups (Fig. 6*C*). It is interesting that islets with a cross-sectional area of 11–20 cells seemed particularly sensitive both to the toxic effects of STZ and to the protective effects of the treatment, displaying marked increases in β-cell fraction and fraction of total pancreatic β-cell number in response to the DP IV inhibitor regimen (Fig. 6*B*, *C*, *I*, and *J*).

Qualitative analysis of insulin immunoreactivity revealed a number of interesting features. Whereas insulin-IR cells in the islets of STZ control animals displayed relatively diffuse and weak staining (Fig. 6*F*), islets in both treatment groups contained a marked number of cells displaying more intense insulin staining (Fig. 6*G* and *H*). These insulin-"bright" (IN^{bright}) cells appeared relatively rotund and polarized, whereas those of STZ control islets appeared diffuse and elongated and lacked distinct polarity (Fig. 6*E*–*J*).

Incretin-mediated protection of β-cells from STZ in vitro. In an attempt to provide a mechanism of action for the immunohistochemical and the in vivo data, an examination was made of the ability of GIP and GLP-1 to promote cell survival in INS-1 (832/13) cells transiently exposed to STZ. After a 30-min STZ exposure followed by a 24-h recovery period, STZ was shown to elicit cytotoxic effects in the low mmol/l range, with an EC_{50} of 2.1 mmol/l (Fig. 7*A*). GLP-1 and GIP were shown independently to reverse STZ-induced cytotoxicity partially with EC_{50} values of 0.04 and 0.75 nmol/l, respectively (Fig. 7*B*). STZ-induced cytotoxicity under control conditions involved

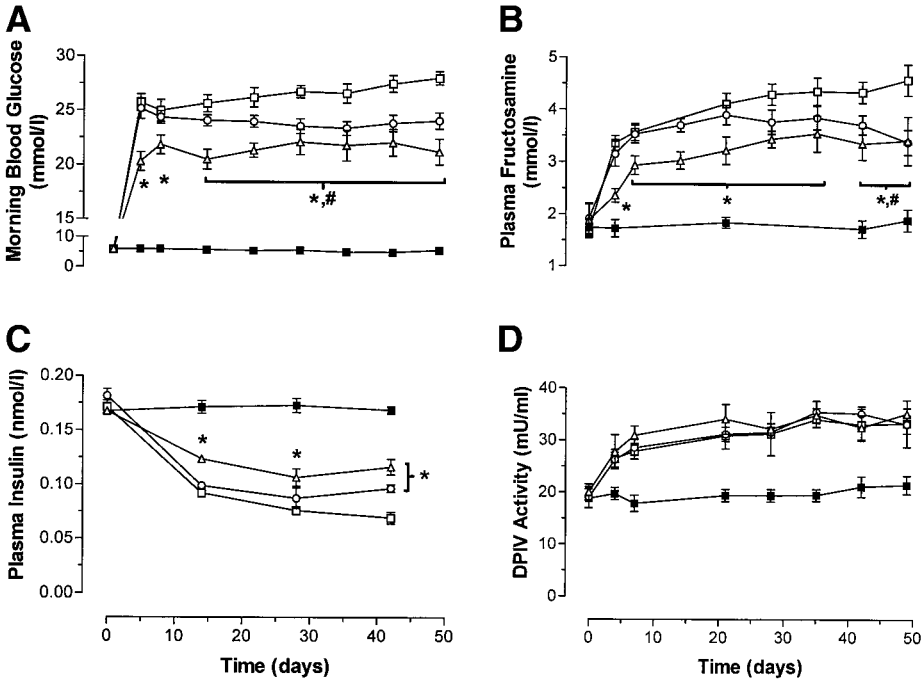

FIG. 3. Weekly monitoring of blood glucose (*A*) and plasma fructosamine (*B*), insulin (*C*), and DP IV activity levels (*D*) in Wistar rats ($n = 20$) exposed to a single high dose of STZ (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Control (solid squares) and STZ control (open squares) animals were administered a 1% cellulose solution, whereas the early treatment group (open triangles; treatment initiated 1 week before STZ administration) and the late treatment group (open circles; treatment initiated 1 week after STZ administration) were administered 10 mg/kg P32/98 twice daily by oral gavage. Blood glucose was measured on a hand-held blood glucose meter, insulin and fructosamine were measured using commercially available kits, and DP IV activity was measured using an in-house assay. *Statistical significance ($P < 0.05$) for the early group versus STZ control; #statistical significance for the late group versus STZ control.

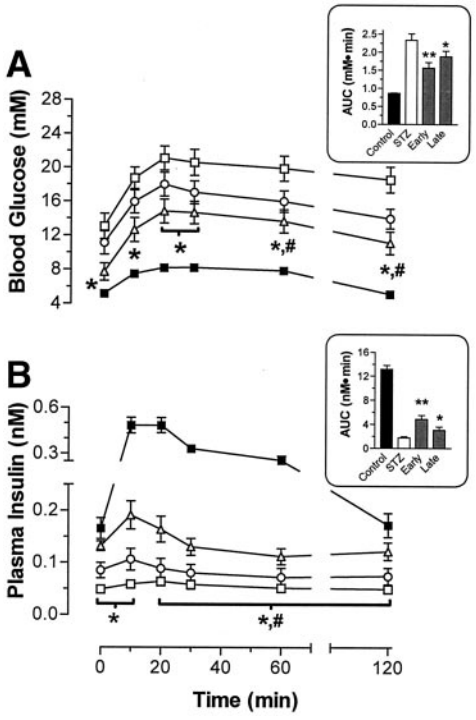

FIG. 4. OGTT performed on Wistar rats ($n = 20$) exposed to a single high dose of STZ (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98 for 7 weeks. Blood glucose (*A*) and plasma insulin (*B*) were measured during a 1 g/kg OGTT in control (solid squares) and STZ control (open squares) animals that were administered a 1% cellulose solution and an early treatment group (open triangles; treatment initiated 1 week before STZ administration) and late treatment group (open circles; treatment initiated 1 week after STZ administration) that received 10 mg/kg P32/98 twice daily by oral gavage for 7 weeks after STZ administration. Insets show area under the curve calculations. *Statistical significance ($P < 0.05$) for the early group versus STZ control; #statistical significance for the late group versus STZ control.

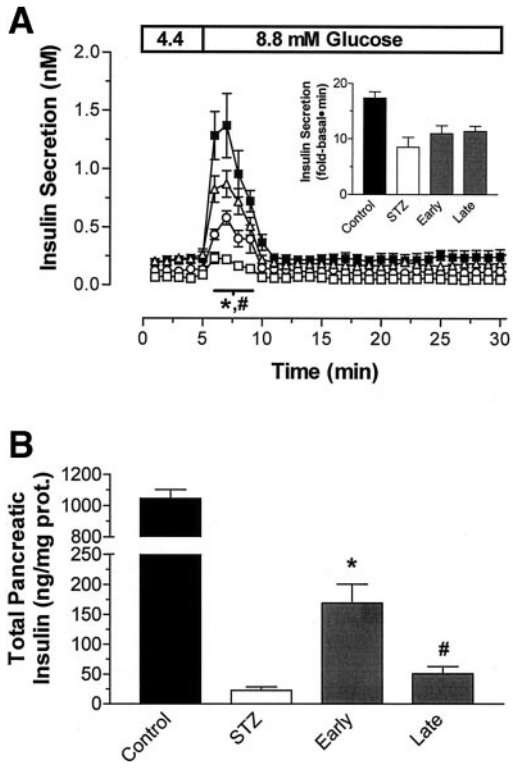

FIG. 5. Insulin response to glucose (*A*) and insulin content (*B*) in pancreata isolated from Wistar rats ($n = 20$) exposed to a single high dose of STZ (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Glucose-stimulated insulin secretion was assessed during an ex vivo pancreas perfusion, and pancreatic insulin content determinations were made for entire pancreata excised under anesthesia. Inset shows area under the curve for the 5th to 10th minutes. *Statistical significance ($P < 0.05$) for the early group versus STZ control; #statistical significance for the late group versus STZ control.

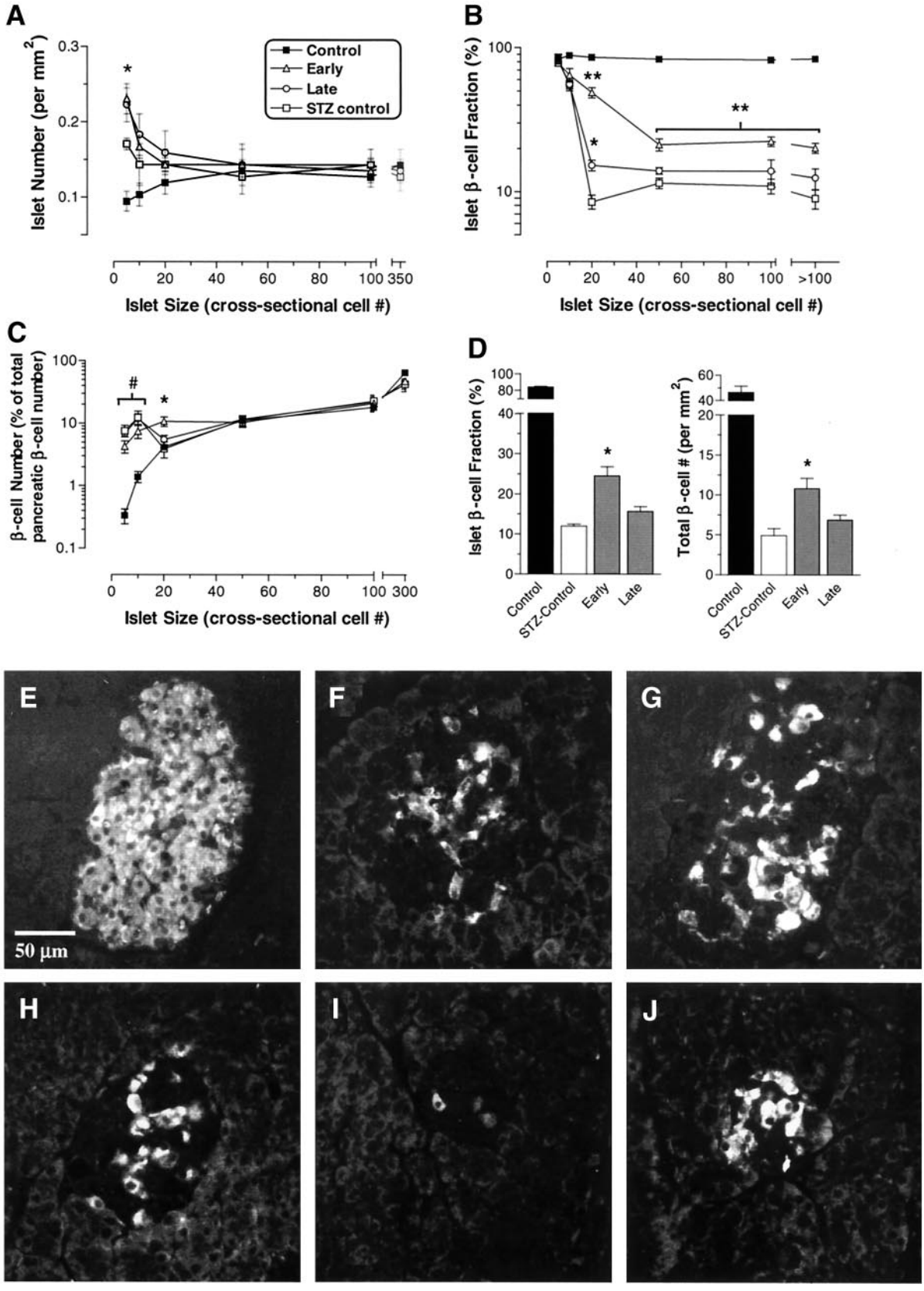

FIG. 6. Immunohistochemical analyses of islet sections stained for insulin. Islets were binned according to cross-sectional endocrine cell number (0–5, 6–10, 11–20, 21–50, 51–100, 100–350) and reported as the largest islet size of any given bin. *A*: Number of islets per unit area. *B*: Fraction of islet endocrine cells immunoreactive for insulin per size bin. *C*: Insulin-IR cells as a fraction of the total number of insulin-IR cells per subject. *D*: β-Cell fraction of total islet endocrine cells combined for all sizes and number of insulin-IR cells per unit pancreatic area. Insulin staining from normal Wistar rat (*E*), and STZ-control (*F*), early P32/98 treated (*G*), and late P32/98 treated (*H*) Wistar rats exposed to 50 mg/kg STZ 7 weeks earlier. Islets that contained ~20 cells in cross-section appeared particularly sensitive to both STZ (*I*) and the protective effects of the DP IV inhibitor treatment (*J*; early treatment group).

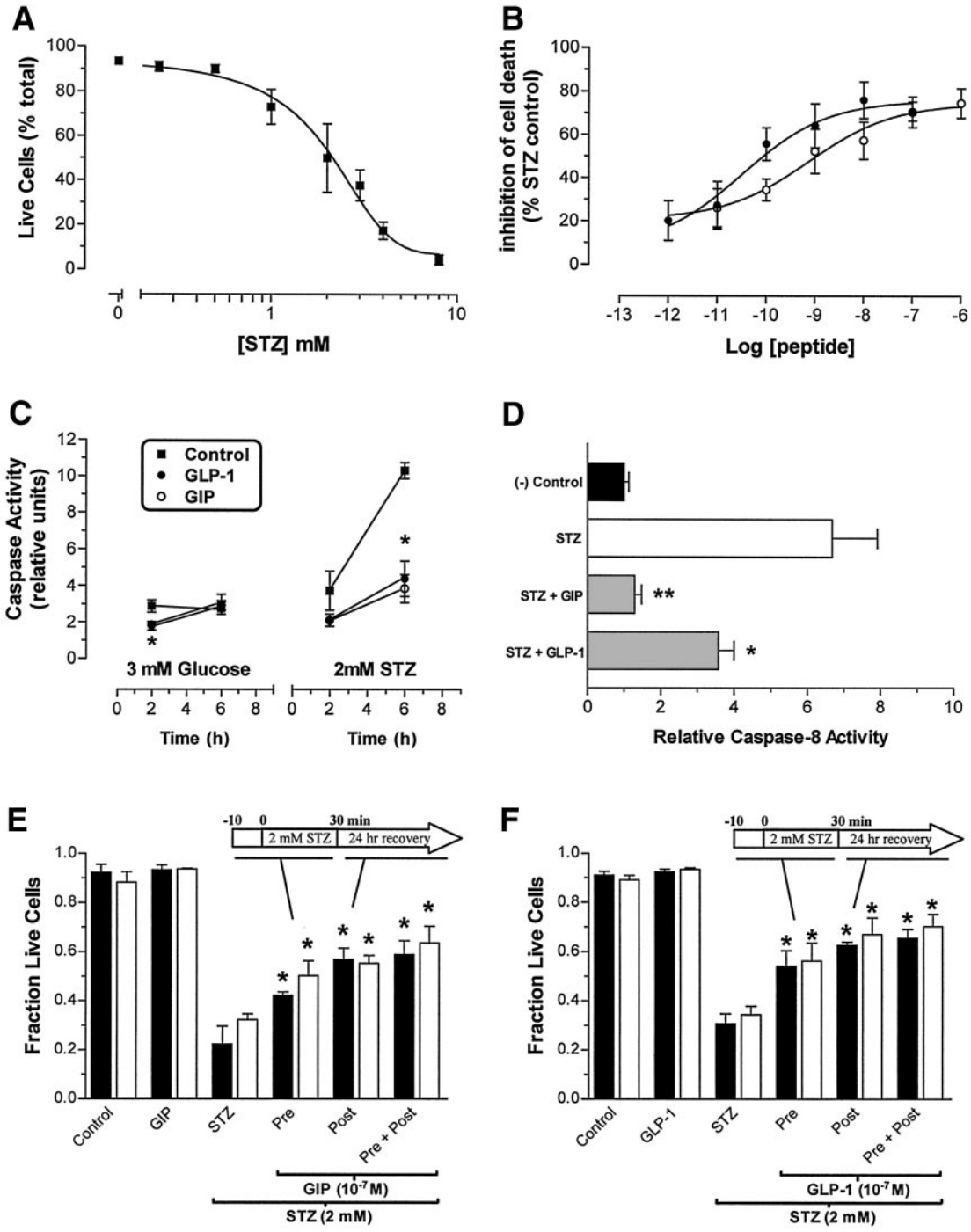

FIG. 7. GIP and GLP-1 stimulation of β-(INS-1 832/13) cells protects against STZ-induced apoptotic cell death. *A*: STZ dose-response curve in INS-1 832/13 cells. *B*: Dose dependence of GIP/GLP-1–mediated inhibition of STZ-induced cell death. *C* and *D*: GIP and GLP-1–mediated suppression of STZ-induced caspase-3 and -8 activation. Additional examination of prevention of STZ-induced cell death by GIP (*E*) and GLP-1 (*F*) in the presence (open bars) or absence (closed bars) of the DP IV inhibitor P32/98 (10 μmol/l) showed no strong dependence on whether peptide was added before (Pre), immediately after (Post), or before and after (Pre + Post) a 30-min STZ (2 mmol/l) exposure.

three- and sevenfold increases in caspase-3 and -8 activity, respectively, increases that were largely ablated by the addition of the incretins (Fig. 7*C* and *D*). Furthermore, only minimal differences in cytoprotection were observed when the interval of GLP-1 administration was varied between pretreatment and recovery treatment (Fig. 7*E*). Addition of GIP and GLP-1 in combination showed no significant additive effect (data not shown).

Effects of DP IV inhibitor treatment on plasma lipids and hepatic PEPCK levels. Table 1 summarizes the results of plasma triglyceride, free fatty acid, and cholesterol measurements made on fasting samples obtained 7 weeks into the experiment. An indication of insulin insufficiency and a characteristic of type 1 animal models, the STZ controls displayed significant increases in circulating levels of all three lipids. Treatment with the DP IV

TABLE 1
Plasma lipids and hepatic PEPCK activity in STZ-exposed Wistar rats treated with the DP IV inhibitor P32/98

	Control	STZ control	Early treatment	Late treatment
PEPCK (mU/kg)	22.7 ± 1.7	46.3 ± 4.3††	24.2 ± 2.4*	35.9 ± 4.8†
Triglycerides (mmol/l)	0.50 ± 0.14	2.37 ± 0.28††	0.94 ± 0.11**†	1.61 ± 0.22*††
NEFAs (mmol/l)	0.28 ± 0.04	0.52 ± 0.05††	0.24 ± 0.03*	0.41 ± 0.02††
Cholesterol (mmol/l)	1.59 ± 0.16	2.53 ± 0.31†	1.54 ± 0.10*	1.75 ± 0.12*

NEFAs, nonesterified fatty acids. Statistical significance vs. STZ controls is indicated by *($P < 0.05$) or **($P < 0.01$) and vs. controls by †($P < 0.05$) or ††($P < 0.01$).

inhibitor P32/98 resulted in a lasting reduction in plasma lipids with the early treatment group returning to control triglyceride and cholesterol levels (Table 1). Another metabolic abnormality characteristic of the STZ rat is an increase in hepatic PEPCK activity. Early treatment with the DP IV inhibitor P32/98 reversed this defect, whereas delayed treatment elicited a nonsignificant, 46% reduction in the STZ-induced rise in PEPCK activity (Table 1).

DISCUSSION

The current study represents the first examination of long-term DP IV inhibition in an animal model of type 1 diabetes. Traditionally, the impetus for research into incretin physiology and into DP IV inhibitor–mediated enhancement of these endocrine axes has been the potential of GIP and GLP-1 not only to stimulate insulin secretion but also to enhance β-cell secretory function (glucose competency and insulin production), parameters altered in type 2 diabetes. Although the potential of exploiting the glucagonostatic actions of GLP-1 in the treatment of type 1 diabetes has also been investigated, research into the therapeutic potential of the incretins toward the autoimmune disorder has been minimal (26–28). Recent studies have added stimulation of β-cell growth and differentiation, and inhibition of β-cell apoptosis to a growing list of "antidiabetic" incretin effects (5–11). It was these latter findings that provided the initial rationale for an investigation into the effects of long-term DP IV inhibition on glucose homeostasis and islet integrity in the STZ model of type 1 diabetes. Through the demonstration of β-cell protection, stimulation of islet neogenesis, and enhancement of overall glucose homeostasis, we show for the first time the potential for DP IV inhibitors as a therapeutic strategy for the treatment of type 1 diabetes.

One hallmark of type 1 diabetes, in both humans and animal models, is limited weight gain and low BMI, direct consequences of insulin insufficiency. Lack of insulin-stimulated nutrient uptake stimulates hyperphagia, whereas hyperglycemia promotes polyuria and subsequently polydipsia. Thus, body weight and food and water intake serve as indicators of proper glucose handling. DP IV inhibitor treatment showed partial reversal of STZ-induced changes in all three metabolic indicators, suggesting improvements in glucose homeostasis and nutrient uptake (Fig. 2). Increased weight gain, concomitant with a reduction in food intake in the treated groups, indicates improved anabolic (likely insulin-dependent) function, a notion supported by weekly morning glucose, insulin, and fructosamine measurements (Fig. 3); OGTT data (Fig. 4); and pancreas perfusions (Fig. 5).

That said, it is important to note that all of these measures (weekly monitoring, OGTT, and pancreas perfusion) were performed well after drug washout (~16 h after the last dose of P32/98) and therefore represent cumulative effects of long-term DP IV inhibition rather than the effects of acute incretin enhancement. The blunting and partial reversal of STZ-induced deviations in weekly glucose and fructosamine and experimentally challenged blood glucose values indicate a marked improvement in oral glucose tolerance in the treated animals (Figs. 3 and 4). Parallel blood glucose profiles during the OGTT, coupled with only minimal enhancement of glucose responsiveness of insulin secretion (Figs. 4*B*, inset, and 5*A*, inset), suggested that the improvements in glucose tolerance were secondary to enhanced total insulin-secretory capacity rather than enhanced insulin sensitivity (Fig. 4). The relatively limited enhancement of β-cell function (responsiveness) evidenced in both the OGTT (Fig. 4*B*, inset) and the pancreas perfusion (Fig. 5*A*, inset) was in contrast to a relatively profound improvement in glucose responsiveness found in a previous study of long-term DP IV inhibition in obese Zucker rats (19). To define further the pancreatic effects of DP IV inhibition in the STZ model and to provide a potential underpinning for the in vivo data, we performed immunohistochemical analyses of pancreatic sections and in vitro cytoprotection studies.

Long-term DP IV inhibitor treatment was shown to both preserve (early treatment) and increase (early and late) β-cell number through an apparent stimulation of islet neogenesis, and β-cell regeneration (differentiation from precursor cells) and/or enhanced insulin biosynthesis (Fig. 6). Evidence for preservation of a population of β-cells, aside from the enhancement of β-cell precursor differentiation, comes largely from the dichotomy of the effects of early versus late treatment with P32/98. All indexes of glucose handling examined showed a profound and immediate improvement in the early treatment group relative to the late treatment group (Figs. 2 and 3). Also, the substantial increases in β-cell number, pancreatic insulin content, and islet β-cell fraction in the early treatment group relative to late provide strong evidence for a cytoprotective effect of DP IV inhibitor treatment on β-cells (Figs. 5 and 6). The potential of the incretins as mediators of these improvements was highlighted by dose-dependent reversal of STZ-induced cell death in the INS-1 (832/13) β-cell line (Fig. 7). These data, which included suppression of aberrant caspase-3 and -8 activity, support an antiapoptotic mechanism of action; however, they do not exclude the potential contribution of incretin effects on cell proliferation. Growth-limiting conditions (serum-free, low-glucose media) were used, however, to minimize proliferation, a strategy that proved effective as evidenced by the lack of change in total cell number between STZ-exposed experimental groups.

Protective effects aside, the immunohistochemical analysis showed evidence for what was likely either enhanced insulin biosynthesis or β-cell regeneration, as well as for islet neogenesis. Evidence for the latter included a significant increase in islets of the smallest size subsets in both DP IV inhibitor–treated groups (Fig. 6*A*). These islets were found to contain a near-normal β-cell fraction (60–85%) and to be composed almost exclusively of intensely stained, morphologically normal β-cells (IN^{bright}). In contrast, larger islets contained a broad range of insulin-IR cells, suggesting their presence during the STZ insult. The increase in number of very small, intensely stained islets in the treated groups is consistent with previous reports of GLP-1–stimulated β-cell differentiation, islet budding, and islet neogenesis (7,29,30)

What was the nature of the IN^{bright} cells contained within the larger, more mature islets? Teitelman and colleagues (31,32) showed in two separate studies the presence of a wave of intra-islet β-cell regeneration/differentiation that peaked within the first few days after high-dose STZ administration in CD-1 mice and disappeared 7–30 days after exposure. These intra-islet β-cell precursor cells were shown to be of PP-cell or δ-cell origin, staining positive for the pancreatic-duodenum homeobox-1 (PDX-1) and either pancreatic polypeptide or somatostatin ($PDX\text{-}1^+/PP^+$ and $PDX\text{-}1^+/SOM^+$, respectively). These $PDX\text{-}1^+/PP^+/IN^+$ and $PDX\text{-}1^+/SOM^+/IN^+$ β-cell precursors were found within 24 h of exposure to high-dose STZ and were shown to be preferentially induced by insulin-dependent normalization of hyperglycemia (31,32). If the IN^{bright} cells observed in the present study are the mature product of these adult β-precursor cells, then it would seem as though DP IV inhibitor treatment, likely through the enhancement of GIP and GLP-1 levels, enhanced the differentiation of intra-islet precursors and/or prolonged their survival in a severely hyperglycemic environment. GLP-1 has been shown to enhance PDX-1 expression in vitro, and in vivo to stimulate differentiation of β-cell precursors within the islets of old glucose-intolerant rats (29). Additional data from the current study that support such a conclusion include *1*) a doubling of pancreatic insulin content despite a minimal increase in insulin-IR cell number in the late treatment (versus STZ-control), *2*) the finding that IN^{bright} cells were detected in the STZ control animals, and *3*) that there was a 1.3-fold increase in islet β-cell fraction in larger islets (>50 cells in cross-section) in the late treatment group versus the STZ controls (Fig. 6). Despite the strength of these findings, the data are still suggestive, and a confident description of the mechanisms underlying the increases in β-cell and islet number and in pancreatic insulin content will require a detailed temporal examination of this model at various stages of treatment.

Notwithstanding that GIP and GLP-1 are discussed as the primary mediators of protective and reparative processes reported above, there remain a number of endogenous DP IV substrates whose enhancement might also play a role (33). For instance, glucagon (34), vasoactive intestinal polypeptide (35), and pituitary adenylate cyclase-activating polypeptide (35) all are relatively good substrates for DP IV and have been shown to play significant roles in the regulation of β-cell function and development (36–38). The contribution of these and other peptides toward the present findings is very likely to be significant. In addition to nonincretin substrate involvement, effects of DP IV inhibitors on immune function (including clearance of apoptotic β-cells) cannot be ruled out as contributing factors toward our findings. DP IV inhibitors have been shown to suppress a number of T-cell–, B-cell–, and NK-cell–specific immune functions (39).

Although the functional relevance of DP IV in the immune system has yet to be elucidated, its definitive involvement makes the investigation of DP IV inhibition in type 1 diabetes intriguing. The potential for combined immunosuppressive and incretin-enhancing effects is a unique therapeutic paradigm. Having described the stimulatory effects of DP IV inhibitors on β-cell survival and regeneration and on overall glucose tolerance in a type 1 model that highlights insulin deficiency, β-cell apoptosis, and β-cell regeneration, this study now warrants an investigation in an autoimmune model of the disease. Although both have their own limitations with respect to autoimmunity, the NOD mouse and BB rat would provide suitable models for a study into DP IV inhibitor effects on the autoimmune aspect of type 1 diabetes.

In summary, we have shown that treatment of STZ-induced diabetic rats with the DP IV inhibitor P32/98 stimulates islet neogenesis and β-cell regeneration, increases pancreatic insulin content, and significantly improves overall glucose tolerance. The findings set the foundation for additional study into the application of DP IV inhibitors in the treatment of insulin-dependent diabetes (type 1 and late-stage type 2) perhaps filling the void for an inexpensive, benign, preventive therapy or to be used in combination with existing therapies (i.e., insulin) after diagnosis.

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