Long-Term Treatment With the Dipeptidyl Peptidase IV Inhibitor P32/98 Causes Sustained Improvements in Glucose Tolerance, Insulin Sensitivity, Hyperinsulinemia, and β-Cell Glucose Responsiveness in VDF (*fa/fa*) Zucker Rats

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The incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are responsible for >50% of nutrient-stimulated insulin secretion. After being released into the circulation, GIP and GLP-1 are rapidly inactivated by the circulating enzyme dipeptidyl peptidase IV (DP IV). The use of DP IV inhibitors to enhance these insulinotropic hormonal axes has proven effective on an acute scale in both animals and humans; however, the long-term effects of these compounds have yet to be determined. Therefore, we carried out the following study: two groups of *fa/fa* Zucker rats ($n = 6$ each) were treated twice daily for 3 months with the DP IV inhibitor P32/98 (20 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}, p.o.). Monthly oral glucose tolerance tests (OGTTs), performed after drug washout, revealed a progressive and sustained improvement in glucose tolerance in the treated animals. After 12 weeks of treatment, peak OGTT blood glucose values in the treated animals averaged 8.5 mmol/l less than in the controls (12.0 ± 0.7 vs. 20.5 ± 1.3 mmol/l, respectively). Concomitant insulin determinations showed an increased early-phase insulin response in the treated group (43% increase). Furthermore, in response to an 8.8 mmol/l glucose perfusion, pancreata from controls showed no increase in insulin secretion, whereas pancreata from treated animals exhibited a 3.2-fold rise in insulin secretion, indicating enhanced β-cell glucose responsiveness. Also, both basal and insulin-stimulated glucose uptake were increased in soleus muscle strips from the treated group (by 20 and 50%, respectively), providing direct evidence for an improvement in peripheral insulin sensitivity. In summary, long-term DP IV inhibitor treatment was shown to cause sustained improvements in glucose tolerance, insulinemia, β-cell glucose responsiveness, and peripheral insulin sensitivity, novel effects that provide further support for the use of DP IV inhibitors in the treatment of diabetes. *Diabetes* 51: 943–950, 2002

In 1995, Kieffer et al. (1) showed glucose-dependent insulinotropic polypeptide-(1–42) (GIP) and glucagon-like peptide 1-(-36)amide (GLP-1) to be substrates of the circulating enzyme dipeptidyl peptidase IV (DP IV) in vivo. DP IV is an ubiquitous ectopeptidase that preferentially cleaves oligopeptides with a penultimate prolyl, alanyl, or seryl residue at the NH$_2$-terminus, a substrate specificity that encompasses a number of bioactive peptides including GIP, GLP-1, and the counterregulatory hormone glucagon (2,3). DP IV−mediated cleavage of GIP and GLP-1 is a rapid process, yielding a circulating half-life of 1–2 min for the parent peptides. The resultant NH$_2$-terminally truncated products GIP$_{3-42}$ and GLP-1$_{9-36amide}$ have been shown in a number of studies to be inactive at the receptor level and thus noninsulinotropic (4,5). Subsequent studies have clearly established DP IV−mediated NH$_2$-terminal truncation as the primary mechanism for GIP and GLP-1 inactivation (1,6–8).

Also known as the incretins, GIP and GLP-1 make up the endocrine component of the entero-insular (gut-pancreas) axis—a concept describing the neural, endocrine, and substrate signaling pathways between the small intestine and the islets of Langerhans (9). Together, the incretins are responsible for >50% of nutrient-stimulated insulin release. In addition, the incretins share a number of non-insulin-mediated effects that contribute to effective glucose homeostasis. GIP and GLP-1 have both been shown to inhibit gastric motility and secretion (10,11), promote β-cell glucose competence (12), and stimulate insulin gene transcription and biosynthesis (13,14). In addition, GIP has been reported to play a role in the

regulation of fat metabolism (15), and GLP-1 has been shown to stimulate β-cell differentiation and growth (16), as well as to restore islet-cell glucose responsiveness (17).

We have previously shown that acute administration of the specific DP IV inhibitor P32/98 (isoleucyl-thiazolidine) in Zucker rats enhances insulin secretion and glucose tolerance (18), improvements that were much more profound in the diabetic, fatty animals than in their lean littermates (19). Balkan et al. (20) confirmed these findings using the DP IV inhibitor NVP-DPP728 and went on to provide evidence for the previously postulated stabilization of, and rise in, plasma active GLP-1$_{7-36}$ (GLP-1a) after inhibitor treatment. However, despite its efficacy, the use of DP IV inhibitors on an acute scale is unlikely to exploit the longer term incretin actions involving altered intracellular protein function and gene expression. It was therefore hypothesized that chronic DP IV inhibitor treatment of diabetic animals, in addition to improving glucose tolerance, would enhance β-cell glucose responsiveness, replication, and turnover, and thus result in sustained improvements in β-cell function.

In the present study, two groups of Vancouver diabetic fatty (VDF) rats were treated for 3 months with the DP IV inhibitor P32/98. VDF rats are a substrain of the fatty (*fa/fa*) Zucker rat, which display abnormalities characteristic of type 2 diabetes, including mild hyperglycemia, hyperinsulinemia, glucose intolerance, hyperlipidemia, impaired insulin secretion, and peripheral and hepatic insulin resistance (21). Parameters including body weight, food and water intake, and oral glucose tolerance were regularly examined to track the progression of the disease and study the possible therapeutic effects of the inhibitor. At the end of the treatment period, ex vivo fat and muscle insulin sensitivity were assessed, and pancreas perfusion was performed to measure β-cell glucose responsiveness. The results provided the first demonstration that long-term DP IV inhibitor treatment causes progressive and sustained improvements in glucose tolerance, insulin sensitivity, and β-cell glucose responsiveness.

RESEARCH DESIGN AND METHODS

Materials. The DP IV inhibitor P32/98 (di-[2S,3S]-2-amino-3-methyl-pentanoic-1,3-thiazolidine fumarate) was synthesized, as previously described (22).

Animals. We randomly assigned six pairs of male fatty (*fa/fa*) VDF Zucker rat littermates to a control or treatment (P32/98) group at 440 g body wt (age 11 ± 0.5 weeks). Animals were housed on a 12-h light/dark cycle (lights on at 0600) and allowed access to standard rat diet and water ad libitum. The techniques used in this study were in compliance with the guidelines of the Canadian Council on Animal Care and were approved by the University of British Columbia Council on Animal Care, Certificate # A99-006.

Protocol for daily monitoring and drug administration. The treatment group received P32/98 (10 mg/kg) by oral gavage twice daily (0800 and 1700) for 100 days, and the control animals received concurrent doses of vehicle consisting of a 1% cellulose solution. Every 2 days, body weight, morning and evening blood glucose, and food and water intake were assessed. Blood samples were acquired from the tail, and glucose was measured using a SureStep analyzer (Lifescan Canada, Burnaby, Canada). Food and water intake were measured by subtraction.

Protocol for monthly assessment of glucose tolerance. Every 4 weeks from the start of the experiment, an oral glucose tolerance test (OGTT; 1 g/kg) was performed after an 18-h fast and complete drug washout (~12 circulating half-lives for P32/93). No 0800 dose was administered in this case. Blood samples (250 μl) were collected from the tail using heparinized capillary tubes, centrifuged, and stored at –20°C. In the case of the 12-week OGTT, blood was collected directly into tubes containing the DP IV inhibitor P32/98 (final concentration 10 μmol/l) for analysis of active GLP-1 (EGLP-35K; Linco

Research, St. Charles, MO). Plasma insulin was measured by radioimmunoassay using a guinea pig anti-insulin antibody (GP-01), as previously described (23), and blood glucose was measured as described above. Plasma DP IV activity was determined using a colorimetric assay measuring the liberation of *p*-nitroanilide (A$_{405\ nm}$) from the DP IV substrate H-gly-pro-*p*NA (Sigma; Parkville, Ontario, Canada). It is important to note that the assay involves a 20-fold sample dilution and therefore underestimates the actual degree of inhibition occurring in the undiluted sample when using rapidly reversible inhibitors such as P32/98.

Estimations of insulin sensitivity made from OGTT data were performed using the composite insulin sensitivity index proposed by Matsuda and DeFronzo (24). Calculation of the index was made according to the following equation:

$$CISI = \frac{10,000}{\sqrt{(FPG \cdot FPI)(MG \cdot MI)}}$$

where FPG and FPI are fasting plasma glucose and insulin concentrations, respectively, and MG and MI are the mean glucose and insulin concentrations, respectively, over the course of the OGTT.

Protocol for 24-h glucose, insulin, and DP IV profile. To determine the effects of DP IV inhibition over a 24-h period, blood glucose, insulin, and DP IV activity levels were measured as described above, every 3 h for 24 h, 6 weeks into the study. Drug dosing was continued at the appropriate times during the profile.

Skeletal muscle insulin sensitivity. Uptake of ^{14}C-labeled glucose in soleus muscle strips was measured as an indicator of skeletal muscle insulin sensitivity. In brief, after an overnight fast and 18 h after the last dose of P32/98, the animals were anesthetized with pentobarbital sodium (Somnotol; ~50 mg/kg). The soleus muscles of both hindlimbs were exposed and isolated. After freeing the muscle by severing the proximal and distal tendons, strips of ~25–35 mg were pulled from the muscle (the two outer thirds of each muscle were used). After being weighed, the strips were fixed onto stainless steel clips at their resting length and allowed to stabilize for 30 min in a Krebs-Ringer bicarbonate buffer supplemented with 3 mmol/l pyruvate, continuously gassed with 95% O_2:5% CO_2 and held at 37°C in a shaking water bath. These conditions were maintained for the duration of the experiment, unless otherwise stated.

To assess glucose uptake in response to insulin, muscle strips underwent two preincubations (30 and 60 min) followed by a 30-min test incubation. Both the second preincubation and the test incubation contained 0 or 800 μU/ml insulin. The test incubation was performed in media supplemented with [^{3}H]inulin (0.1 μCi/ml) as a measure of extracellular space and the nonmetabolizable glucose analogue [^{14}C]-3-*O*-methylglucose (0.05 μCi/ml) for measurement of glucose uptake. After incubation, each strip was blotted dry and digested with proteinase K (0.25 μg/ml), and the radioactivity of the muscle digests was measured with a liquid scintillation−counting dual-isotopic program.

Adipose tissue insulin sensitivity. To estimate insulin sensitivity in adipose tissue, glycogen synthase (GS) and acetyl-CoA carboxylase (ACC) levels were measured, as previously described (25,26). In brief, 3-cm^3 samples of ependymal adipose tissue were obtained from anesthetized animals and subjected to a 16-min collagenase digestion (0.5 mg/ml). Recovered adipocytes were washed three times and allowed to stabilize for 1 h in 37°C Krebs buffer repetitively gassed with 95% O_2:5% CO_2. Then 2-ml aliquots of the adipocyte suspension containing 0, 100, 250, 800, and 1,500 μU/ml insulin were incubated for 30 min and immediately flash frozen on liquid nitrogen and stored at –70°C. Before ACC and GS assessment, stored samples were thawed, homogenized in buffer (pH 7.2) containing 20 mmol/l MOPS, 250 mmol/l sucrose, 2 mmol/l EDTA, 2 mmol/l EGTA, 2.5 mmol/l benzamidine, and centrifuged (15 min at 15,000*g*).

For measurement of ACC activity, 50-μl aliquots of supernatant, preincubated in the presence or absence of 20 mmol/l citrate, were added to 450 μl of [^{14}C]HCO$_3$ containing assay buffer (pH 7.4; 50 mmol/l HEPES, 10 mmol/l MgSO$_4$, 5 mmol/l EDTA, 5.9 mmol/l ATP, 7.8 mmol/l glutathione, 2 mg/ml BSA, 15 mmol/l KHCO$_3$, 150 μmol/l acetyl CoA). After 3 min, the reaction was arrested by the addition of 200 μl of 5 mol/l HCl. Samples were dried for 6 h, resuspended in 400 μl of distilled water, combined with 3 ml scintillation cocktail, and counted on a Beckman LS 6001C β-counter.

GS activity was measured using a modification of a filter paper method (26): 25 μl of the cell extracts, prepared as indicated above, were added to assay buffer (pH 7.0; 75 mmol/l MOPS, 75 mmol/l NaF, 10 mg/ml glycogen, 2 mmol/l UDP-[^{14}C]glucose) held at 30°C in the presence or absence of 15 mmol/l glucose-6-phosphate. Each reaction was stopped by spotting 50 μl of the reaction mixture onto Whatmann 3MM filter paper and immersing the paper in 66% ethanol. After three ethanol washes, the samples were air dried

and the $[^{14}C]$ activity (UDP-$[^{14}C]$glucose incorporation into glycogen) was determined.

Protocol for pancreas perfusion. After excision of soleus and ependymal adipose tissue samples, the pancreas was isolated and perfused with a low-to-high glucose (4.4 to 8.8 mmol/l) perfusion protocol, as previously described (27). After exposure through a mid-line incision on the ventral aspect, the pancreas was isolated, all minor vessels were 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.

Immunohistochemistry and β-cell mass determination. Pancreata were removed from anesthetized animals (50 mg/kg sodium pentobarbital) and placed directly into fixative for 48 h (44% formaldehyde, 47% distilled H_2O, 9% glacial acetic acid). After being embedded in paraffin, 5-μm tissue sections were cut, mounted onto slides, and dried ready for staining. To assess β-cell area, sections were stained first with a guinea pig anti-insulin primary antibody and then with peroxidase-conjugated goat anti-guinea pig secondary. Slides were developed using diaminobenzidine and counterstained with hematoxylin. Analyses were performed using Northern Eclipse Software (Empix Imaging, Mississauga, Ontario, Canada), as previously described (28).

Statistical Analysis. Student's t test and ANOVA were used, where appropriate, to test statistical significance of the data ($P < 0.05$). Analyses were performed using Prism 3.0 data analysis software (GraphPad Software, San Diego, CA).

RESULTS

Effects of P32/98 treatment on body weight, daily blood glucose, and food and water intake. VDF rats treated with P32/98 displayed a 12.5% (25 g) reduction in weight gain over the 3-month treatment period (control: 211 ± 8 g; treated: 176 ± 6 g) (Fig. 1A). Measurements of food and water intake revealed a minor decrease in water intake (Fig. 1B) in the treated animals concomitant with unaltered food intake. Food intake over the course of the experiment averaged 30.0 ± 0.4 and 30.4 ± 0.3 g · rat^{-1} · day^{-1} in the treated and control groups, respectively. Food and water intake decreased over the course of the experiment, paralleling the decrease in the rate of weight gain as the growth of the animals began to plateau at around 600–650 g (data not shown). Twice-daily monitoring of blood glucose revealed no differences in morning or evening blood glucose values between the experimental groups, although neither group displayed notably hyperglycemic values (data not shown). Morning blood glucose levels over the course of the experiment averaged 5.0 ± 0.1 and 5.3 ± 0.1 mmol/l in the treated and control animals, respectively. Evening blood glucose values averaged 6.7 ± 0.1 and 7.0 ± 0.2 mmol/l, respectively. Hematocrit, measured at 4-week intervals, indicated no adverse effects of the blood-sampling protocol used, averaging 43.4–45.3% in both groups.

Effects of P32/98 treatment on blood glucose, insulin, and DP IV levels over 24 h. After 6 weeks of treatment, a 24-h profile of blood glucose, insulin, and DP IV activity levels was obtained by taking blood samples at 3-h intervals, interrupting neither treatment administration nor the light/dark cycle. The profile confirmed that administration of P32/98 caused significant inhibition of DP IV activity over the majority of the 24-h cycle, with at least 65% inhibition during the feeding cycle (Fig. 2A). The integrated blood glucose excursion in the treated animals was 75% that of the controls, peaking at 7.7 ± 0.3 mmol/l, as compared to 9.8 ± 0.6 mmol/l for the untreated animals (Fig. 2B). The corresponding plasma insulin profile showed a decrease not only in peak insulin values, but also in "basal," nonfeeding values (~0800 to 1800) in the treated animals (Fig. 2C).
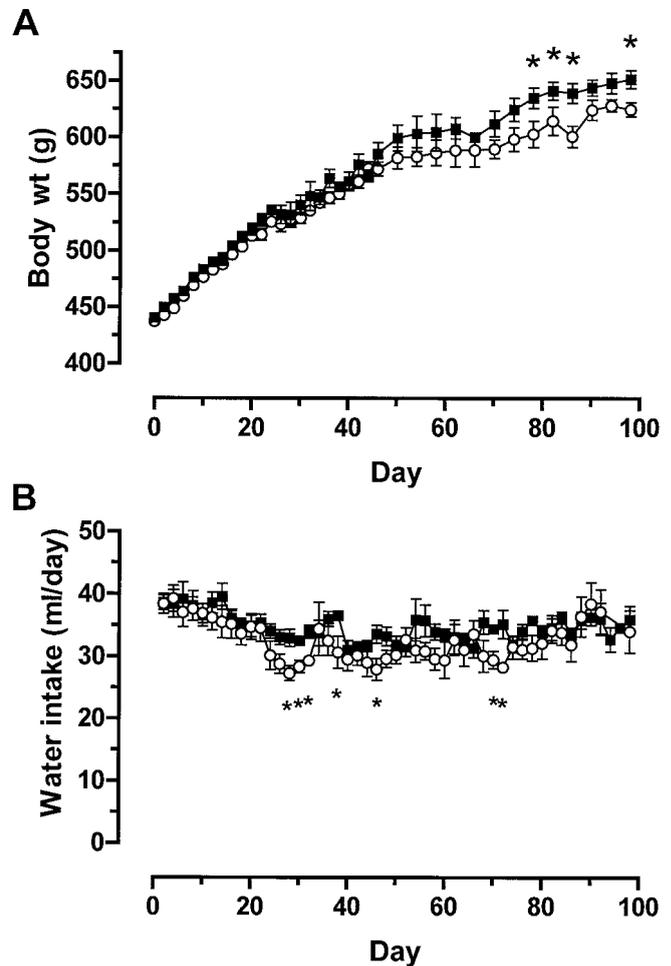

FIG. 1. Body weight and water intake measured in DP IV inhibitor−treated (○) or control (■) VDF rats ($n = 6$ in each group). Body weight (A), and water intake (B) were measured along with morning and evening blood glucose levels and food intake (not shown) every 2 days. *$P < 0.05$ vs. other group.

Effects of P32/98 treatment on oral glucose tolerance. The three OGTTs, performed in the absence of circulating P32/98 and at 1-month intervals, were used to monitor the progression of the disease state in the control animals and to document any improvements displayed in the treated group. The initial OGTT, administered after 4 weeks of treatment, showed significant decreases (~2 mmol/l) in basal and 45-, 60-, and 90-min blood glucose values in the treated group, despite overlapping plasma insulin excursions (Fig. 3A). Data from the second OGTT were very similar, with the exception that the 120-min blood glucose value was also significantly lowered in the treated group than in the control group (10.8 ± 0.8 vs. 12.3 ± 0.8, respectively); once again, the insulin profiles were superimposable (data not shown). The final OGTT, performed after 12 weeks of treatment, showed a marked difference in glucose tolerance between the two groups, with significantly decreased blood glucose values observed at all time points. Peak blood glucose values in the treated group averaged 12.0 ± 0.7 mmol/l, 8.5 mmol/l less than that of control animals (Fig. 3B), whereas 2-h values in the treated group had returned to 9.2 ± 0.5 mmol/l, a 40% reduction compared to control values. GLP-1a levels, measured during the final OGTT using an NH_2-terminally
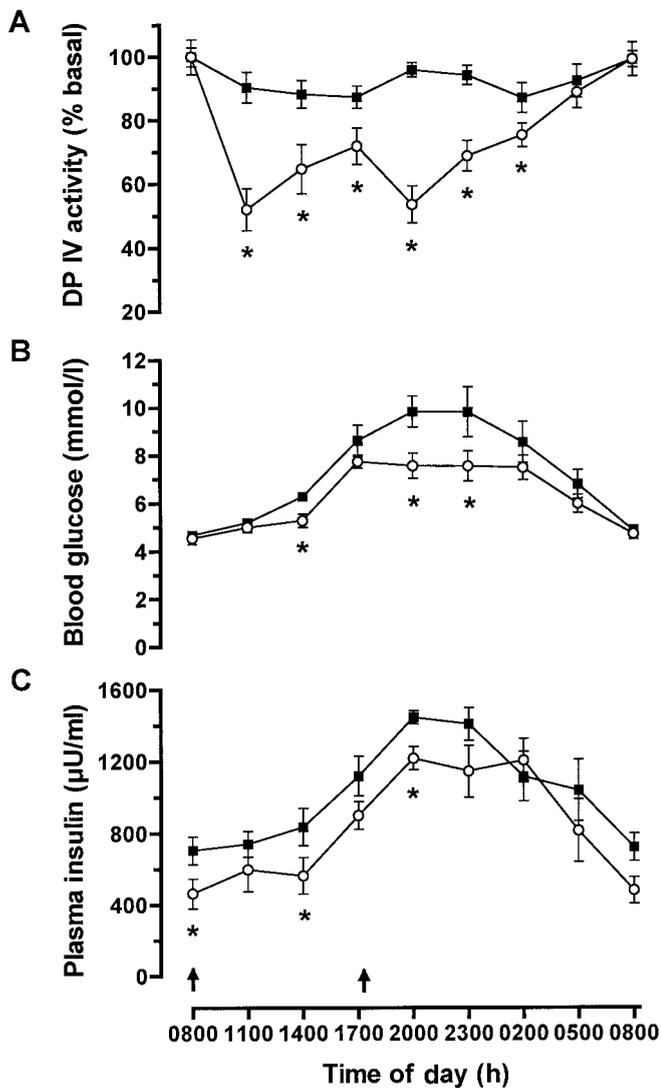
FIG. 2. A 24-h profile of plasma DP IV activity (*A*), blood glucose (*B*), and plasma insulin (*C*) levels in VDF rats after 6 weeks of treatment, either with (○) or without (■) the DP IV inhibitor P32/98 (*n* = 6 in each group). Treated animals were administered 10 mg/kg P32/98 twice daily, as indicated by the arrows, and whereas the control group received only the 1% cellulose injection vehicle. *P < 0.05 vs. other group.

directed enzyme-linked immunosorbent assay, were found to be unchanged (Fig. 3*B*). Despite this lack of altered GLP-1a levels, the early-phase insulin response measured in the treated group exceeded that of the control animals by 43%. However, the integrated insulin responses between the two groups showed no significant differences. Analysis of the OGTT data using the composite insulin sensitivity index of Matsuda and DeFronzo (24) revealed a progressive increase in estimated insulin sensitivity of the treated animals relative to controls (Fig. 3*C*).

Comparison of the OGTTs over the course of the experiment revealed a progressive decrease in both fasting and peak blood glucose values in animals treated with P32/98, improvements that were not observed in control animals (Fig. 4*A* and *B*). Peak insulin values did not differ significantly between the two experimental groups until the final, 12-week, OGTT, at which time the peak insulin levels in the treated animals exceeded those of the control

animals by an average of 43% (Fig. 4*C*). Plasma DP IV activity, measured at the start of each OGTT, was significantly increased in the treated group by week 8 of the study, and the elevation was maintained at week 12 (Fig. 4*D*).

Effects of chronic DP IV inhibitor treatment on pancreatic glucose responsiveness. A low-to-high step glucose perfusion protocol was performed on the pancreata of half of each group of animals. The shift from 4.4 to 8.8 mmol/l glucose perfusate caused a 3.2-fold increase in insulin secretory rate in the pancreata from the treated animals (Fig. 5). The insulin secretory rate shifted from a basal 570 ± 170 to over 2,100 μU/min within 2 min of high glucose perfusion. The same glucose step procedure failed to elicit any significant response in the control pancreata until well over 20 min of high glucose perfusion (Fig. 5).

Effects of chronic DP IV inhibitor treatment on muscle and fat insulin sensitivity. To further define the apparent improvements in insulin sensitivity observed in the OGTT data, assays of muscle and fat insulin sensitivity were performed. GS and ACC activity were measured in isolated adipocytes along with uptake of ^{14}C-labeled glucose into soleus muscle strips. ACC levels in adipose from both experimental groups were minimal (approaching limits of detection), lacked insulin responsiveness, and showed no differences between the two groups (data not shown). GS activity also appeared insensitive to insulin, although the activity of the enzyme at all measured insulin concentrations was higher in the treated animals than in their control littermates (Fig. 6*A*). Soleus muscle strips taken from the treated animals exhibited significantly higher rates of glucose uptake, in both the basal and the insulin-stimulated states. Glucose uptake in the nonstimulated state was 22% higher in the treated rats (Fig. 6*B*). The insulin-stimulated rise in glucose uptake was enhanced in the treated compared to in the control group (87.5 ± 10.4 vs. 58.5 ± 3.5 cpm/mg tissue at 800 μU/ml insulin, respectively).

Effects of chronic DP IV inhibitor treatment on β-cell area and islet morphology. The 3-month oral DP IV inhibitor regimen yielded no significant differences in β-cell area or islet morphology. Islets from control and treated animals comprised 1.51 ± 0.04 and 1.50 ± 0.03% of the total pancreatic area, respectively. Large, irregularly shaped islets with significant β-cell hyperplasia were observed in both groups, a morphology characteristic of the *fa/fa* Zucker rat.

DISCUSSION

The use of DP IV inhibitors to enhance the entero-insular axis has attracted much recent interest as a potential therapeutic strategy in the treatment of diabetes. Several recent studies have established the efficacy of these compounds on an acute scale (18–20,29). However, investigations performed on an acute scale do not exploit the potential benefits of long-term incretin effects, such as the enhancement of β-cell glucose sensitivity and the stimulation of β-cell mitogenesis, differentiation, and insulin biosynthesis. Here we reported the results of the first study of long-term DP IV inhibitor treatment in a model of type 2 diabetes, the VDF *fa/fa* rat. The results demonstrated that long-term DP IV inhibition arrested the progression of the
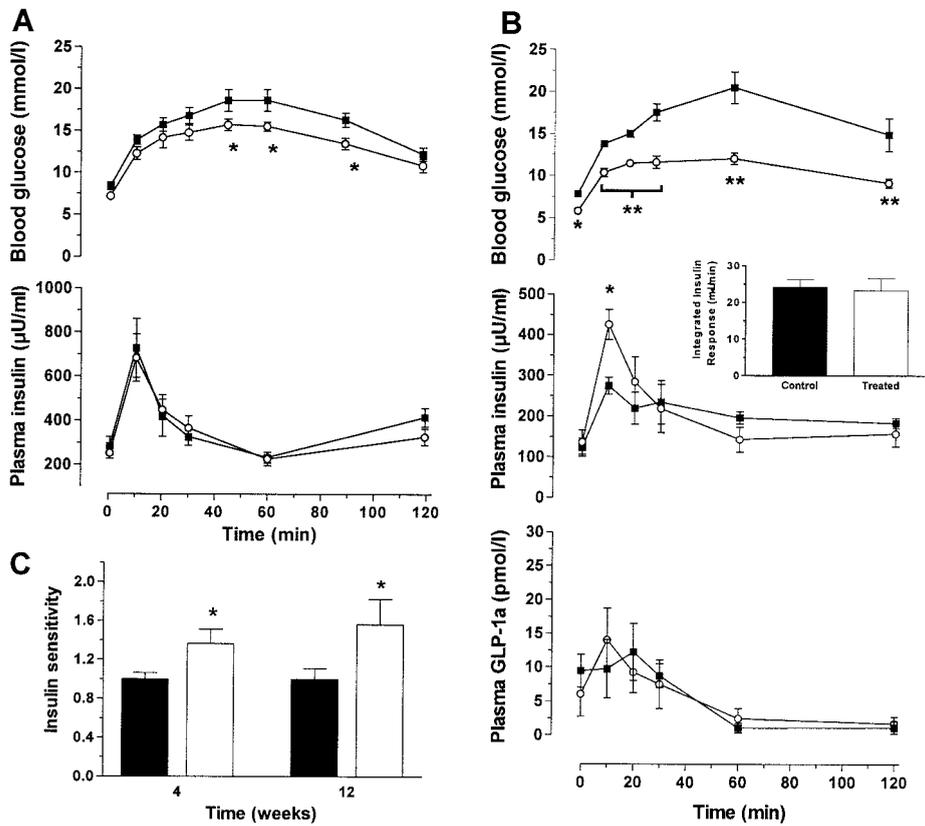
FIG. 3. OGTT administered to both DP IV inhibitor−treated (○) and control (■) VDF rats (*n* = 6 in each group) after 4 (*A*) and 12 (*B*) weeks of treatment. Blood glucose and plasma insulin measurements were performed in both series of tests, while the active fraction of plasma GLP-1 was also measured at 12 weeks. The inset in B shows the integrated plasma insulin responses for the 12-week OGTT. *C*: Relative insulin sensitivity, control versus treated, corresponding to the 4- and 12-week OGTTs shown in *A* and *B*. *P < 0.05 vs. other group.

fa/fa Zucker diabetic syndrome and caused a progressive improvement in glucose tolerance, insulin sensitivity, and β-cell glucose responsiveness.

Daily monitoring revealed a 12.5% decrease in body weight gain (4% reduction in final body weight) in the treated animals compared to untreated controls (Fig. 1*A*).
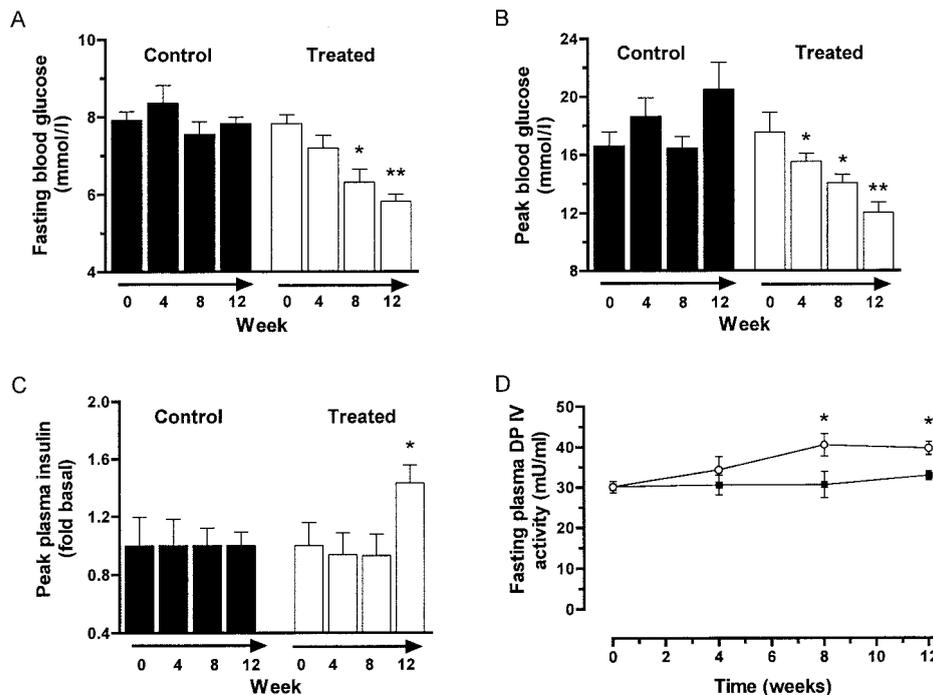

FIG. 4. Comparison of fasting (*A*) and peak (*B*) blood glucose, peak plasma insulin (*C*), and fasting plasma DP IV activity (*D*) measured during OGTTs performed at 4-week intervals in control (■) or DP IV inhibitor−treated (○) VDF rats (*n* = 6 in each group). *P < 0.05 vs. other group.
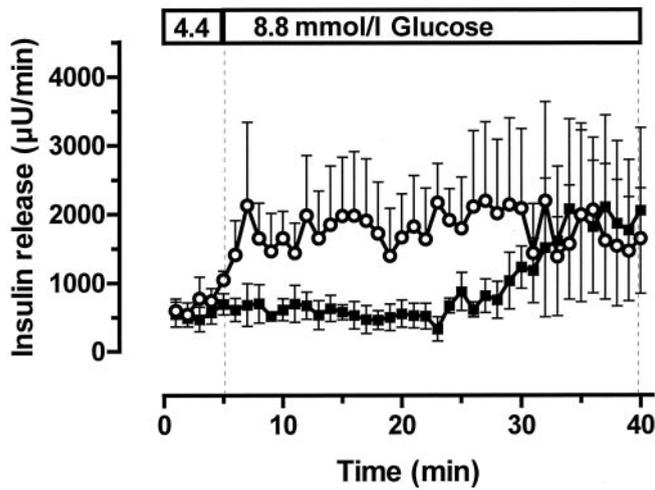
FIG. 5. Insulin release measured during perfusion of pancreata from VDF rats after 3 months of treatment with (○) or without (■) the DP IV inhibitor P32/98 (*n* = 3 in each group).

Although not statistically significant, mean food intake in the treated animals averaged 0.4 g · day^{-1} · rat^{-1} (41 g/rat over the course of the study) less than those in the control
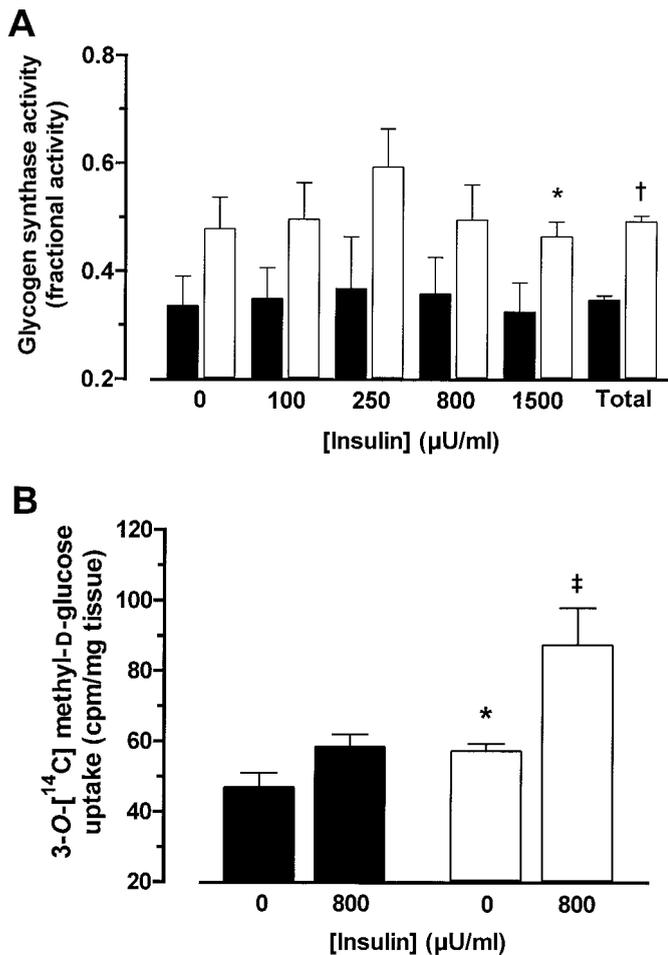

FIG. 6. Adipose tissue glycogen synthase activity (*A*) and uptake of 3-*O*-[¹⁴C] methyl-D-glucose into soleus muscle strips (*B*) isolated from VDF rats after 12 weeks of P32/98 treatment (□) or a control 1% cellulose solution (■) (*n* = 6 in each group). *P < 0.05 vs. corresponding control value; †P < 0.01 vs. corresponding control value; ‡statistically significant difference from basal ([insulin] = 0 μU/ml).

group. The cumulative 41 g/rat nonsignificant difference in food intake over the course of the experiment might partially account for the decreased weight gain in the treated animals. These findings rule out neither the possibility that the gastric inhibitory actions of the incretins nor the reported satiety effects of GLP-1 played a role in the decrease in weight gain.

Monitored every 2 days, morning and evening blood glucose values showed no significant response to the inhibitor treatment, a likely reflection of two points. First, the blood-sampling times (0800 and 1700) corresponded to postabsorptive and early feeding states, respectively, with blood glucose values in the ranges of 4.5–5.5 and 6.0–8.0 mmol/l, respectively. In light of the hypothesized, glucose-dependent mechanism of action of the treatment, large decreases in glucose values would not be anticipated at these glycemic levels. Second, both morning and evening blood samples were collected immediately before drug dosing, at times of minimum DP IV inhibition, when the potential for any acute therapeutic effects of the treatment were at a minimum. Both points are supported by the 24-h profile shown in Fig. 2.

The unaltered postabsorptive blood glucose values notwithstanding, DP IV inhibitor treatment effectively reduced both prandial blood glucose and blood glucose responses to an OGTT (Figs. 2 and 3). During the 24-h profile, the control animals exhibited a 105% rise in plasma insulin in response to a 5.2-mmol/l increase in blood glucose, whereas the treated animals displayed a 160% insulin response to a much smaller glucose excursion (3.0 mmol/l). Although these differences were likely attributable, at least in part, to an acute increase in circulating incretin levels induced by P32/98, the pronounced early-phase insulin peak exhibited during the OGTT was not (the OGTT took place after complete drug washout). The latter data suggest not only an increased insulin sensitivity, but also an enhanced β-cell glucose responsiveness, in treated animals. Ultimately, an increase in β-cell glucose responsiveness was clearly demonstrated through pancreas perfusion. After exposure to an elevated (8.8 mmol/l) glucose perfusate, pancreata from the control animals showed an absence of first-phase insulin release, whereas those from the treated group exhibited an immediate 3.2-fold insulin response (Fig. 5). The absence of an early-phase insulin release seen in the control group is characteristic of the VDF rat and is a hallmark of type 2 diabetes (21). Considering the lack of altered β-cell area or islet morphology, these data suggest that long-term treatment with P32/98 causes an improvement in the ability of the existing β-cell population to sense and respond to increases in glucose concentration. These findings are consistent with the reported effects of GLP-1 on β-cell differentiation, as well as numerous reports showing the glucose-sensitizing effects of GIP and GLP-1 in both islets and immortalized β-cell models (30,31).

Elevated fasting blood glucose in the face of hyperinsulinemia and poor clearance of an oral glucose load are consistent with the hepatic and muscle insulin resistance, respectively, described in the *fa/fa* Zucker rat. Findings in the present study showed that DP IV inhibitor treatment at least partially corrected both of these metabolic deviations, suggesting improvements in both sites of insulin

resistance. An increased glucose-to-insulin ratio evident during the postabsorptive state of the 24-h profile (Fig. 2), as well as fasting values of the 12-week OGTT (Figs. 3 and 4), were consistent with a decrease in insulin resistance in the treated animals. The latter increase in insulin sensitivity was shown to be significant at both 4 and 12 weeks using the composite insulin sensitivity index of Matsuda and DeFronzo (24). This mathematical analysis was previously validated (with high correlation) against the hyperinsulinemic-euglycemic clamp technique in 153 subjects with varying degrees of insulin resistance. The relative insulin sensitivity of the treated animals improved with each successive OGTT, ultimately reaching a relative index score 1.56 ± 0.26 times that of the control animals. The results of the 24-h glucose/insulin/DP IV profile and the OGTT were corroborated by direct measurements of glucose uptake in soleus muscle strips, which clearly demonstrated improved glucose uptake in both the non-stimulated and the insulin-stimulated states (Fig. 6). Though somewhat controversial, both GIP and GLP-1 (and exendin-4) have been reported to increase muscle insulin sensitivity through the stimulation of glycogen synthesis and glucose uptake (32–35). In addition, a number of whole animal studies using GLP-1 or related GLP-1 receptor agonists have observed similar improvements in glucose tolerance and insulin sensitivity. Young et al. (36) showed that long-term administration of the GLP-1 agonist exendin-4 causes glucose-lowering and insulin-sensitizing effects in a number of diabetic animal models, including the *fa/fa* Zucker rat. Also, a number of subchronic infusion studies have revealed improvements in glycemic control, glucose tolerance, and insulin sensitivity (37–39). Our findings are consistent with the results of these previous investigations. However, the indirect contributions of a long-term improvement in glycemia or long-term enhancement of a number of other DP IV substrates (in particular, the insulin secretagogues vasoactive intestinal peptide, pituitary adenylyl cyclase-activating peptide, gastrin-releasing peptide, and neuropeptide Y) over the course of the treatment cannot be ruled out as a causative factor for the metabolic improvements observed. Further experiments will be required to address this issue and that of the relative effect of DP IV inhibitor therapy on each of the major insulin-sensitive tissues.

An important facet shared by the OGTT, the muscle glucose uptake and the pancreas perfusion protocols, was that cessation of drug treatment occurred 18 h before these experimental procedures. Any divergence between groups, therefore, reflected long-term, lasting changes in metabolic state, rather than an acute effect of the drug. Drug washout was confirmed by DP IV activity measurements. It is interesting that an increase in DP IV activity was observed in the treated animals over the course of the study, which was likely a compensatory response to chronic inhibition of DP IV activity in the treated group (>90% of the 24-h cycle). The significance of this finding is not fully understood, as the circulating, soluble form of DP IV measured in the present study represents only 5–10% of the entire DP IV pool (2). Therefore, further investigation into the effects of DP IV inhibitor therapy on other sources of DP IV activity in the circulation (particularly lymphocytic and endothelial DP IV) is warranted. It is likely that the compensatory change in circulating DP IV levels could be avoided by once-daily treatment and/or a lower inhibitor dosage. Therapeutic dosages required to improve glucose tolerance on an acute scale in humans (~0.2 mg/kg) are 100-fold lower than those used in the present study (40,41).

Several therapeutic strategies exploiting the antidiabetic effects of GIP and GLP-1 are currently being pursued. The success of DP IV inhibition is owed in part to a mechanism of action that impacts only a single physiological regulatory system. DP IV inhibition slows the rate of incretin inactivation, but leaves the nutrient-dependent mechanism of incretin release intact and the glucose dependence of their anti-diabetic actions unaltered. The present study showed for the first time that long-term DP IV inhibitor therapy leads to enhanced peripheral insulin sensitivity and β-cell glucose responsiveness, improvements that culminate in markedly improved glucose tolerance well beyond the clearance time of the drug. Further, the findings of this study exemplify the importance of the noninsulinotropic effects of GIP and GLP-1 in the regulation of β-cell function. In conclusion, the data presented here establish the potential utility of DP IV inhibitors in the treatment of diabetes.

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