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Chronic Inhibition of Circulating Dipeptidyl Peptidase IV by FE 999011 Delays the Occurrence of Diabetes in Male Zucker Diabetic Fatty Rats

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Acute suppression of dipeptidyl peptidase IV (DPP-IV) activity improves glucose tolerance in the Zucker fatty rat, a rodent model of impaired glucose tolerance, through stabilization of glucagon-like peptide (GLP)-1. This study describes the effects of a new and potent DPP-IV inhibitor, FE 999011, which is able to suppress plasma DPP-IV activity for 12 h after a single oral administration. In the Zucker fatty rat, FE 999011 dose-dependently attenuated glucose excursion during an oral glucose tolerance test and increased GLP-1(7-36) release in response to intraduodenal glucose. Chronic treatment with FE 999011 (10 mg/kg, twice a day for 7 days) improved glucose tolerance, as suggested by a decrease in the insulin-to-glucose ratio. In the Zucker diabetic fatty (ZDF) rat, a rodent model of type 2 diabetes, chronic treatment with FE 999011 (10 mg/kg per os, once or twice a day) postponed the development of diabetes, with the twice-a-day treatment delaying the onset of hyperglycemia by 21 days. In addition, treatment with FE 999011 stabilized food and water intake to prediabetic levels and reduced hypertriglyceridemia while preventing the rise in circulating free fatty acids. At the end of treatment, basal plasma GLP-1 levels were increased, and pancreatic gene expression for GLP-1 receptor was significantly upregulated. This study demonstrates that DPP-IV inhibitors such as FE 999011 could be of clinical value to delay the progression from impaired glucose tolerance to type 2 diabetes. *Diabetes* 51:1461–1469, 2002

Dipeptidyl peptidase IV (DPP-IV) (CD26, EC.3.4.14.5) is a membrane-bound and circulating serine protease with a restricted substrate specificity, hydrolyzing peptides after a penultimate NH_2-terminal proline or alanine residue (1). Mice or rats bearing null mutation of the DPP-IV gene have increased glucose tolerance (2,3). Acute inhibition of DPP-IV activity by specific inhibitors such as isoleucine-thiazolidide, NVP-DPP728, or valine-pyrrolidide has been recently shown to significantly reduce glucose excursion in Zucker fatty (*fa/fa*) rats and high fat–fed glucose-intolerant mice during oral or intraduodenal glucose tolerance tests (4–6). Whatever the animal model used, insulin secretion following glucose administration was enhanced by DPP-IV inhibition, and this has been attributed to the role played by DPP-IV in the early inactivation of the two incretins glucagon-like peptide (GLP)-1 and gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP) (7). These gastrointestinal peptides are released postprandially from intestinal L-cells and duodenal K-cells, respectively (8), and potentiate glucose-induced insulin secretion by sensitizing pancreatic β-cells to stimulation by glucose (9–11). Inactivation of these peptides can be successfully prevented by DPP-IV inhibitors, leading to potentiation of their biological activity (12).

The Zucker fatty rat is obese and insulin resistant, with normal or slightly elevated glucose concentrations, reflecting only mild glucose intolerance. Obesity in these animals is due to a mutation in the leptin receptor gene (13). Like the Zucker fatty rat, the Zucker diabetic fatty (ZDF) rat displays glucose intolerance, marked insulin resistance, and hyperlipidemia, but only the ZDF rat becomes overtly diabetic after 8 weeks of age if fed a diet containing 6.5% fat (14). In the prediabetic state, the male ZDF rat experiences a steady increase in basal insulinemia and plasma free fatty acid (FFA) levels. Hyperglycemia develops between 8 and 10 weeks of age, leading to overt diabetes and collapsing insulin secretion (15). This is similar to the progressive loss of glucose-stimulated insulin secretion in human type 2 diabetes, and thus ZDF rats represent a good animal model for this form of human diabetes (16). Interestingly, GLP-1 retains its potency of enhancing glucose-stimulated insulin release in prediabetic and diabetic ZDF rats (17,18), and chronic administration of exendin-4, a peptide showing agonistic activity at the GLP-1 receptor, demonstrated antidiabetic efficacy in these animals, improving glycemic control and insulin sensitivity (19). Similarly, in type 2 diabetic patients, GLP-1 has been successfully used to normalize fasting and prandial glycemia (20–23). Recently, it has been shown that GLP-1 and analogs also stimulate growth and differentiation of β-cell progenitor cells in the pancreas and may therefore restore

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AFC, 7-amino-4-trifluoromethylcoumarin; DPP-IV, dipeptidyl peptidase IV; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; IC_{50}, half-maximal inhibitory concentration; IDGTT, intraduodenal glucose tolerance test; OGTT, oral glucose tolerance test; RT, reverse transcription; TG, triglyceride.

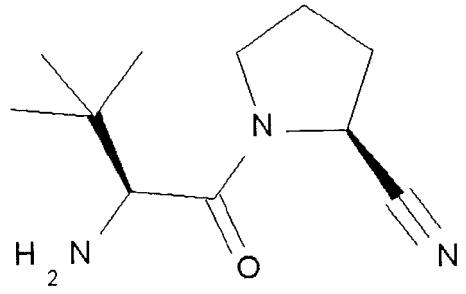

FIG. 1. Chemical structure of FE 999011.

functional β-cell mass when administered to individuals with diabetes (24,25). Taken together, these observations suggest that inappropriate incretin action at the level of the islet cells could be one important factor participating in the development of diabetes. We thus postulated that by chronically increasing endogenous GLP-1 bioactivity in the ZDF rat through constant inhibition of DPP-IV, a similar improvement in glycemic control could be obtained.

In the present study, we have investigated the effects of chronic administration of the new DPP-IV inhibitor FE 999011 [(2S)-1-([2′S]-2′-amino-3′,3′dimethyl-butanoyl)-pyrrolidine-2-carbonitrile] (Fig. 1) in obese Zucker fatty and ZDF rats. FE 999011 is a reversible DPP-IV inhibitor, inhibiting human and rat DPP-IV with half-maximal inhibitory concentration (IC_{50}) values of 7 and 3 nmol/l, respectively (26).

RESEARCH DESIGN AND METHODS

Animals. Male Zucker fatty rats were purchased from Iffa-Credo (L'Arbresle, France) at 8–10 weeks of age; housed in a temperature-, humidity-, and light-controlled room (21–23°C, 12–12 h light-dark cycle); and given free access to food and water. Obese male ZDF rats (n = 24) and lean controls (n = 8), received at 5 weeks of age from Genetic Models (Indianapolis, IN), were individually housed. They were fed ad libitum with Purina 5008 (6.5% fat).

Experimental protocols concerning the use of laboratory animals were reviewed by the University of Geneva School of Medicine Ethics Committee for Animal Experimentation and approved by the State of Geneva Veterinary Office.

Effects of FE 999011 and NVP-DPP728 on plasma DPP-IV activity in the Zucker fatty rat. Three groups of fed male Zucker fatty rats, 11 weeks of age, received an oral administration of FE 999011 or NVP-DPP728 at 10 mg/kg or vehicle (sterile distilled water). Blood samples (200–250 μl) were collected before dosing and then 10, 30, and 60 min and 3, 6, 10, 12, and 24 h postdosing. Tail blood was collected in heparinized tubes (30 units/ml), and plasma was extracted after centrifugation at 3,000 rpm for 10 min and stored at −20°C until determination of DPP-IV activity.

Effects of FE 999011 on glucose excursion during an oral glucose tolerance test orally in the Zucker fatty rat. Four groups of overnight-fasted male Zucker fatty rats, 19–20 weeks of age, were injected with FE 999011 (1, 3, or 10 mg/kg) or vehicle (sterile distilled water). Eight hours later, all animals were administered oral glucose (1 g/kg) as a 40% solution (wt/vol). Blood samples (200–250 μl) were collected before dosing and 10, 20, 30, 60, and 120 min after dosing.

Effects of FE 999011 on the glucose excursion and plasma levels in GLP-1 and insulin following intraduodenal glucose tolerance test in the Zucker fatty rat. Two groups of overnight-fasted obese male Zucker rats and one group of lean rats, 19–20 weeks of age, were anesthetized with pentobarbital (Narcoren, 5 mg/100 g body wt i.p.). A cannula was quickly inserted into the right jugular vein, and the abdomen was exposed by laparotomy. In one obese group of rats, FE 999011 prepared in distilled water was injected intravenously (3 mg/kg), whereas the other rats received a vehicle injection. Fifteen minutes later, a 40% glucose solution (2.5 ml/kg) was injected into the first loop of the duodenum. Blood samples (300 μl) were collected before the glucose load and 4, 7, 10, 13, 16, 20, 30, 60, and 90 min thereafter. For plasma determination of glucose and insulin, 100 μl was taken in heparinized tubes; for plasma determination of GLP-1[(7-36), (7-37)], 200 μl was taken in Eppendorf tubes containing EDTA and diprotin A (Sigma, St. Louis, MO) to achieve a final concentration of 2 mg EDTA and 50 μmol diprotin A per milliliter of blood.

Chronic effects of FE 999011 on glucose and insulin excursion during an oral glucose tolerance test in the Zucker fatty rat. Two groups of male Zucker fatty rats, 15 weeks of age, received FE 999011 (10 mg/kg per os, twice a day) or vehicle (sterile distilled water) during 7 days. Seventeen hours after the final administration of FE 999011, an oral glucose tolerance test (OGTT) was performed as described above, with or without coadministration of FE 999011.

Chronic effects of FE 999011 in the ZDF rat. At 6 weeks of age, obese (*fa/fa*) rats were weighed and randomized into three groups of 8. Two groups were treated with FE 999011 orally once a day (10 mg/kg, 6:00 P.M.) or twice a day (10 mg/kg, 8:00 A.M and 6:00 P.M.), and one group was injected orally with sterile distilled water. A group of eight lean (*fa*/+ or +/+) rats was included as controls for metabolic and molecular parameters. Tail blood was collected from rats in the fed state into heparinized tubes three times a week before the first daily treatment with FE 999011, for plasma determinations of glucose, insulin, triglycerides (TGs), and FFAs. Body weight and food and water consumption were recorded throughout the experiment. The day before the rats were killed, blood samples were taken to determine plasma GLP-1 levels in the fed state. At the end of the study, the rats were killed, and trunk blood was collected in heparinized tubes for plasma determination of DPP-IV activity. Inguinal and retroperitoneal adipose tissues were dissected out and weighed. Pancreata were dissected out and frozen for GLP-1 receptor mRNA measurements.

Plasma analyses. Glycemia was assayed by using a standard glucose-oxidase technique (Boehringer Mannheim, Mannheim, Germany). Plasma insulin concentration was determined by radioimmunoassay (Linco Research, St. Charles, MO). Plasma GLP-1 concentration was determined by an enzyme-linked immunosorbent assay (ELISA) technique that measured the biologically active GLP-1 forms GLP-1(7-36) and GLP-1(7-37) amide in plasma (Linco Research). Plasma TG and FFA concentrations were measured using an enzymatic colorimetric test (Triglyceride L-Type and NEFA C; Wako Chemicals, Richmond, VA).

Plasma DPP-IV activity was measured using a fluorometric assay. The plasma samples were centrifuged at 4°C, 10,000*g*, for 1 min; 0.5 μl supernatant was added to 49 μl assay buffer (HEPES 83.3 mmol/l, EDTA 2 mmol/l, pH 7.8, with 1.5% BSA); and the reaction was started by addition of 0.5 μl substrate Ala-Pro-AFC (7-amino-4-trifluoromethylcoumarin, 20 μmol/l final concentration; Enzyme Systems Products, Livermore, CA) in assay buffer. The initial rate of AFC release was measured at 20°C over 3 min using a Labsystems Fluoroskan Ascent FL instrument at 410 nm excitation/510 nm emission.

Gene expression assays using competitive quantitative RT-PCR. Expression of the gene encoding GLP-1 receptor (GLP-1R) was evaluated by competitive RT-PCR. Tissue was quickly dissected, frozen in liquid nitrogen, then stored at −80°C until processing using a rotor-stator homogenizer. RNA was extracted by using the Qiagen RNeasy Mini Kit method, and then checked for purity and quantified using ultraviolet spectroscopy.

RNase-resistant competitor RNA was synthesized for this gene using the RT-PCR Competitor Construction Kit (Ambion, Austin, TX) and supplied protocol. Using PCR with P3 and P4 primers specific for the gene, a cDNA template was created that was identical to the mRNA but contained the T7 RNA polymerase promoter sequence on the 5′ end, ~50 bp of additional sequence on the 3′ end for efficient reverse transcription (RT) priming, and an internal deletion of 10% to allow for agarose gel size separation of the competitor- and mRNA-derived PCR products. A trace amount of [^{32}P]GTP was added to the synthesis reaction to allow for accurate quantification of the acrylamide gel–purified, full-length competitor.

RT of RNA was done under standard conditions using Moloney murine leukemia virus RT (Life Technologies, Rockville, MD) and random decamer priming. Sample RNA was reverse-transcribed together with known amounts of synthetic RNA competitor. Both the gene-specific and competitor-derived cDNAs were then coamplified using PCR with a specific primer pair for each gene of interest, P1 and P2. PCR was done under standard conditions using *Taq* polymerase (Sigma), prebound with TaqStart antibody (Clontech, Palo Alto, CA) to increase specificity of the reaction. A PE9700 thermocycler (PE Biosystems, Foster City, CA) was used for 30 cycles. A touchdown procedure for primer annealing was used. Cycles consisted of denaturation at 94°C for 10 s; primer annealing at 65 to >60°C for 15 s; and extension at 72°C for 30 s. Primer pairs were designed to span at least one intron so that any PCR products derived from contaminating genomic DNA were easily distinguished from RNA-derived products. Also, PCR extension times were minimized to further reduce the chance of amplification of longer, genomic-DNA derived products.

Two PCR products were obtained for each reaction, and mRNA content was quantified by comparing the amount of DNA in each product band.

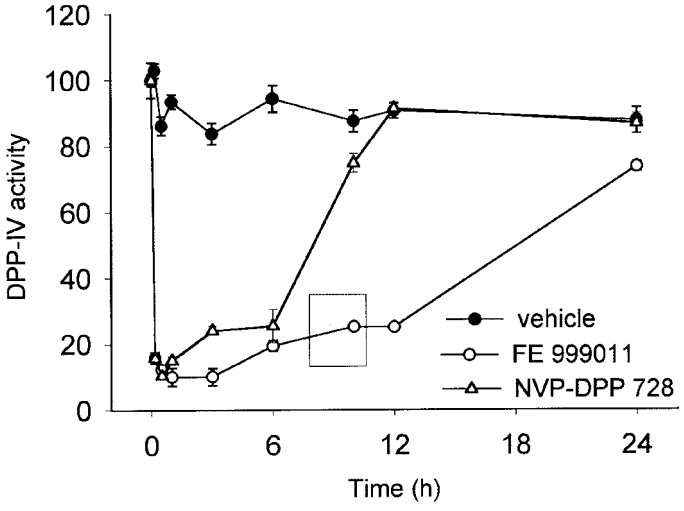

FIG. 2. Effect of FE 999011 and NVP-DPP728 on plasma DPP-IV activity in the Zucker fatty rat. Plasma DPP-IV activity was determined after oral administration of 10 mg/kg FE 999011 (○), 10 mg/kg NVP-DPP728 (△), or vehicle (●). The rectangle indicates when the OGTT was performed as illustrated in Fig. 3. Data are the means ± SE, $n = 6$ rats per group.

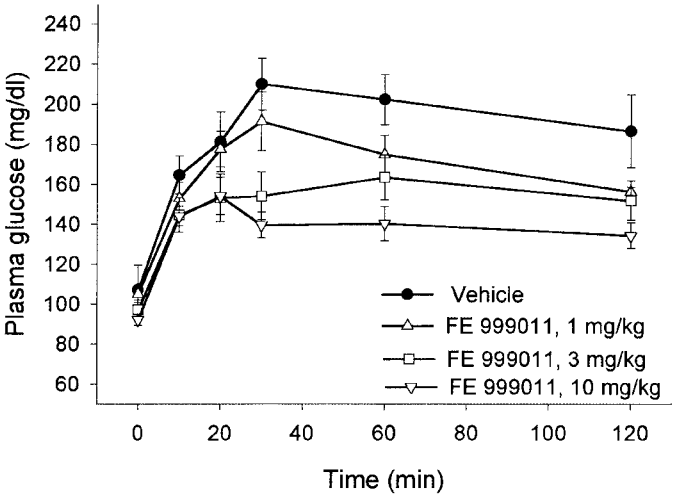

FIG. 3. Effect of previous oral administration of the DPP-IV inhibitor FE 999011 on glucose tolerance in the Zucker fatty rat. Plasma glucose excursion was assessed in response to oral glucose loading (1 g/kg) in the absence (●) or presence of three dosages (1 [△], 3 [□], and 10 [▽] mg/kg) of FE 999011 administered 8 h before. Data are the means ± SE, $n = 6$ rats per group.

Competitor- and mRNA-derived product sizes were 202 and 225 bp, respectively. Coamplification and comparison using another standard (e.g., 18S RNA) is not required for quantification using this technique. Amplified DNA samples were prestained by adding SYBR green dye (Molecular Probes, Eugene, OR) to the loading buffer and electrophoresed on a 3% agarose gel. Gels were analyzed using blue light epi-illumination with a Fuji LAS-1000 digital camera system and quantified using Aida 1D software (Raytest, Wilmington, NC).

The sequences of the primers used are as follows:

GLP-1R P1: 5′-AAT CGG GGT CAA CTT CCT TGT CTT CAT-3′;

GLP-1R P2: 5′-GTG AAG GAG AGC TCT GTG AAC AGC TTG-3′;

GLP-1R P3: 5′-GCG TAA TAC GAC TCA CTA TAG GGA GAG GAG AAT CGG GGT CAA CTT CCT TGT CTT CAT GCC AAG CTG AAG GCT AAT CTC ATG TGT-3′;

GLP-1R P4: 5′-GAC AAA GCA GTA CAA GAC AGC CAC CAT-3′.

Genotyping of rats for leptin receptor mutation. Genomic DNA was extracted from 50–200 μl whole blood using the Qiagen QIAamp DNA Blood Mini Kit and amplified by PCR using primers flanking the nucleotide substitution in the leptin receptor coding sequence that creates the *fa* mutation (5′ primer, 5′-CGT ATG GAA GTC ACA GAT GAT GGT AAT-3′; 3′ primer, 5′-CCT CTC TTA CGA TTG TAG AAT TCT CT-3′). The resulting 118-bp product was digested with *Msp*I and electrophoresed on a 3% agarose gel. Amplicons from the normal allele are not digested and migrate as a single 118-bp band, whereas the mutant allelle is cleaved, resulting in 39- and 79-bp fragments, thus allowing for unambiguous genotyping.

Statistics. All data are presented as means ± SE. They were subjected to ANOVA followed by Student-Newman-Keuls test. $P < 0.05$ was considered significant.

RESULTS

Studies in Zucker fatty rats

Plasma DPP-IV activity following FE 999011 and NVP-DPP728 administration. Oral administration of FE 999011 and NVP-DPP728 to Zucker fatty rats produced an immediate suppression of plasma DPP-IV activity (Fig. 2). Maximal inhibition was obtained 30 min postinjection for NVP-DPP728 and 1 h postinjection for FE 999011. FE 999011 and NVP-DPP728 significantly reduced DPP-IV activity for at least 12 h and 6 h, respectively ($F_{26,45} = 58.763$, $P < 0.001$). Twenty-four hours postinjection, plasma DPP-IV activity in rats treated with FE 999011 had returned to control values.

Effect of FE 999011 on glucose excursion during an OGTT. Pretreatment 8 h before the glucose load with FE 999011 at doses ranging from 1 to 10 mg/kg produced a dose-dependent reduction in plasma glucose excursion during the OGTT (Fig. 3). At the time when the glucose load was given, circulating DPP-IV activity was still suppressed (rectangle in Fig. 2). The integrated glucose responses were 10,046 ± 410 mg · dl^{-1} · 120 min^{-1} for the vehicle group and 7,643 ± 1,032, 6,821 ± 747, and 5,522 ± 644 mg · dl^{-1} · 120 min^{-1} for the groups treated with 1, 3, and 10 mg/kg, respectively. The observed decrease in integrated glucose response by graded doses of FE 999011 was highly significant ($F_{3,20} = 6.57$, $P < 0.01$).

Effect of FE 999011 on glucose excursion, plasma GLP-1, and insulin responses during an intraduodenal glucose tolerance test. A bolus intraduodenal glucose load (1 g/kg) was given to anesthetized lean or obese Zucker rats. One group of obese rats was pretreated with FE 9999011 (3 mg/kg intravenously) 15 min before the glucose load. A steady increase in glycemia was seen in all three groups, with a peak at 20 min for the lean group followed by a slow decrease. The glucose excursion in untreated obese rats was much larger, with a peak at 30 min (area under the curve 3,520.9 ± 382.6 vs. 2,684.0 ± 531.3 mg · dl^{-1} · 60 min^{-1} in lean controls). Glucose excursion in obese Zucker rats pretreated with FE 999011 was indistinguishable from that of lean controls (Fig. 4). The insulin rise in lean controls peaked at 2.1 ± 0.5 ng/ml at 30 min. In untreated obese Zucker rats, insulinemia rose within 20 min from 4.7 ± 2.1 to 13 ± 2.5 ng/ml. Upon treatment with FE 999011, this insulin rise was enhanced, with a peak value at 10 min and a plateau at ~25 ng/ml, persisting until 30 min. Basal values for plasma GLP-1 were indistinguishable between lean and obese untreated rats (2 pmol/l), with no visible increase during the intraduodenal glucose tolerance test (IDGTT). Conversely, pretreatment with FE 999011 produced a brisk rise in plasma GLP-1, with a peak at 12 min (Fig. 4).

Effects of chronic treatment with FE 999011 on glucose excursion and insulin response during an OGTT. The glucose excursion and insulin response of the

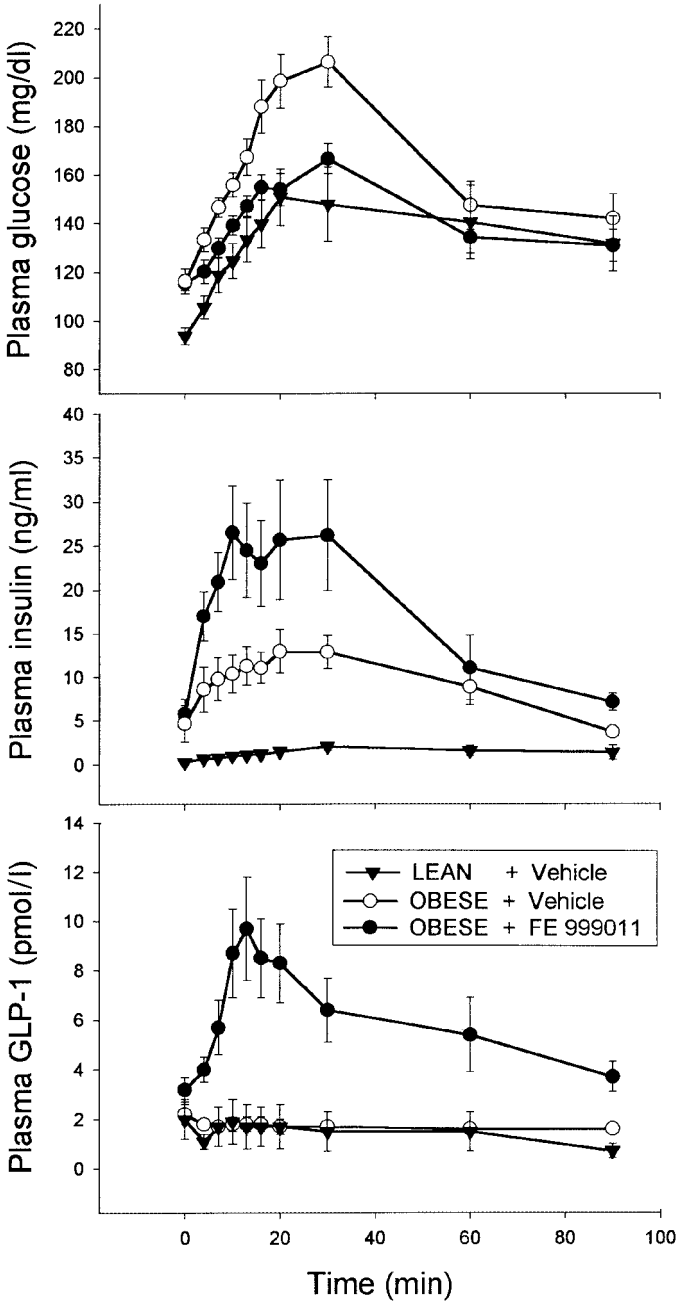

FIG. 4. Effect of previous intravenous administration of the DPP-IV inhibitor FE 999011 on glucose excursion, and plasma insulin and GLP-1 responses during an IDGTT in the Zucker fatty rat. Plasma glucose, insulin, and GLP-1 excursions were assessed in response to an intraduodenal glucose load (1 g/kg) in the absence or presence of 3 mg/kg FE 999011 administered intravenously 15 min before. The ELISA technique used for the measurement of bioactive GLP-1 equally detects GLP-1(7-36) and GLP-1(7-37) amide moieties but none of the inactive forms of GLP-1. Data are the means ± SE, n = 8 rats per group.

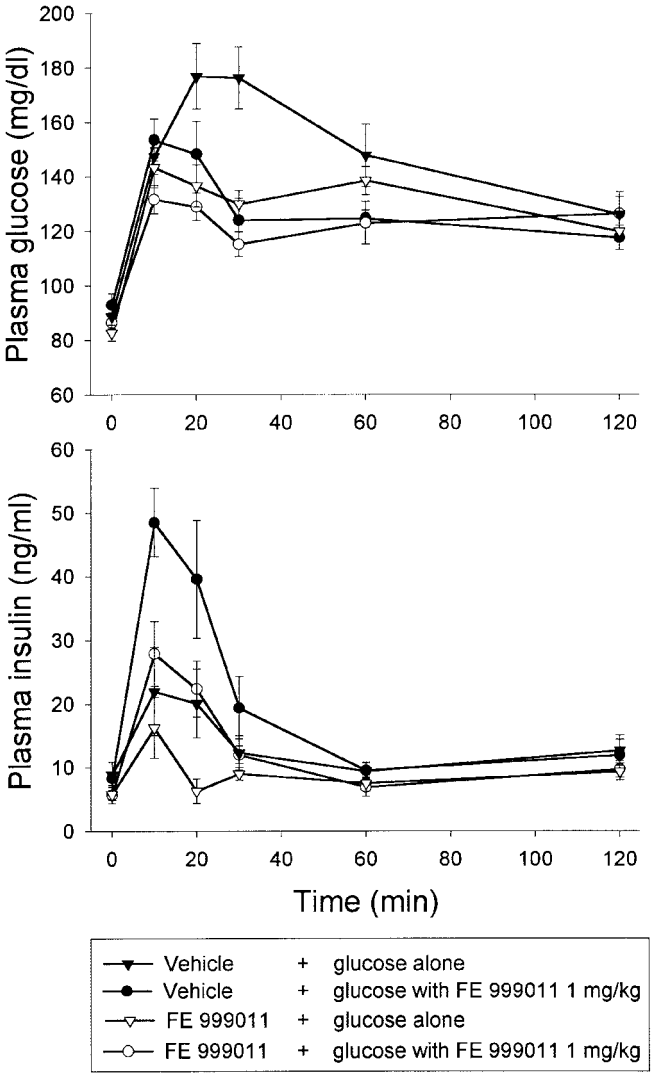

FIG. 5. Effect in the Zucker fatty rat of repeated oral administration for 7 days of FE 999011, 10 mg/kg twice a day (▽, ○), or vehicle (▼, ●) on glucose excursion and insulin response during an OGTT (1 g/kg) performed on the eighth day. At the time of oral glucose administration, rats received either FE999011 (1 mg/kg) with glucose (○, ●) or glucose alone (▽, ▼). Data are the means ± SE, n = 8 rats per group.

control rats that were pretreated with vehicle are shown in Fig. 5 (▼); they exhibit the typical pattern of glucose intolerance known for naive Zucker fatty rats. The addition of FE 999011 (1 mg/kg) to the glucose load in rats that were pretreated with vehicle improved glucose tolerance and enhanced insulin secretion (Fig. 5 [●]). Pretreatment with FE 999011 significantly improved glucose tolerance even when no inhibitor was added to the glucose load (Fig. 5 [▽]). Such improvement in glucose tolerance compared with vehicle-treated rats (▼) was possible with a lower insulin response, suggesting improved insulin sensitivity after 7 days of treatment. Basal insulinemia was decreased in FE 999011 (5.8 ± 0.8 ng/ml) compared with vehicle-treated rats (8.6 ± 1.1 ng/ml, $P < 0.05$). Combination of chronic treatment with FE 999011 for 7 days and addition of FE 999011 to the glucose load produced the lowest glucose excursion (Fig. 5 [○]). In comparison to the rats challenged with glucose alone (▽, ▼), glucose excursion was reduced in rats previously chronically treated with FE 999011 ($F_{11,60} = 14.4$, $P < 0.001$), glycemia at 20 and 30 min postglucose being significantly decreased in the FE 999011–treated group (▽ vs. ▼). The insulin responses were also reduced in rats repeatedly treated with FE 999011 ($F_{11,60} = 2.607$, $P < 0.01$). Insulin-to-glucose ratios were 0.12 ± 0.04 and 0.04 ± 0.01 for rats previously treated with vehicle and FE 999011, respectively. Chronic treatment with FE 999011 had no effect on food intake, water intake, or body weight gain.

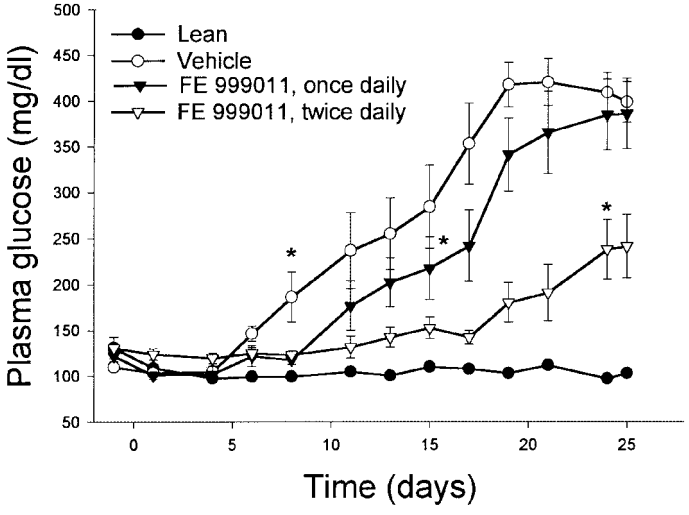

FIG. 6. Pattern of changes in glycemia in lean rats (●), and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day in the evening (▼) or twice a day, morning and evening (▽). In this model, untreated ZDF rats become diabetic between 6 and 10 weeks of age. At time 0, rats were 6.5 weeks old. Data are the means ± SE, n = 8 rats per group.

Studies in male ZDF rats. The leptin receptor mutation was confirmed by genotyping all obese ZDF rats. Lean rats carried either the heterozygote *fa*/+ or the homozygote +/+ genotype.

Pattern of changes in glycemia. At the beginning of the study, glycemia in lean and obese rats was not different (Fig. 6). Eight days later, the obese control group developed hyperglycemia, with plasma levels for glucose of 186.4 ± 27.1 mg/dl in obese rats and 99.4 ± 0.9 mg/dl in lean rats ($P < 0.05$). At day 19, plasma glucose in the control obese group reached a plateau at 417.9 ± 24.1 mg/dl. Treatment with FE 999011 once or twice a day significantly delayed this rise in glycemia in the obese ZDF rats. Plasma glucose levels became significantly increased over lean rat values at day 15 for the once-a-day treated group (217.8 ± 34.0 vs. 110.1 ± 1.9 mg/dl, $P < 0.05$), but only at day 24 for the twice-a-day treated group (238.3 ± 32.4 vs. 97.3 ± 1.6 mg/dl, $P < 0.05$). Overall changes in plasma glucose levels over the 26 days of treatment with FE 999011 (Fig. 6) were highly significant ($F_{51,364} = 21.967$, $P < 0.001$). At the end of the study, glycemia remained highly significantly decreased by the twice-a-day treatment (241.7 ± 34.7 mg/dl) compared with the untreated obese group (399.2 ± 22.4 mg/dl, $P < 0.001$).

Pattern of changes in body weight gain and food and water intake. At the end of the experiment, the cumulative body weight gain was similar for the three obese groups (Fig. 7), but throughout the course of the experiment, body weight gain was differentially affected by treatment ($F_{63,448} = 302.371$, $P < 0.001$). The lean control group gained less weight than the obese groups. From day 11 of treatment, FE 999011 twice a day significantly stabilized weight gain. There was no significant difference between the control obese group and the obese group treated with FE 999011 once a day at any time point. Retroperitoneal and inguinal fat masses were increased in obese compared with lean rats. Treatment with FE 999011 had no effect on fat pad weight. Food consumption in the four groups clearly differed throughout the time course of

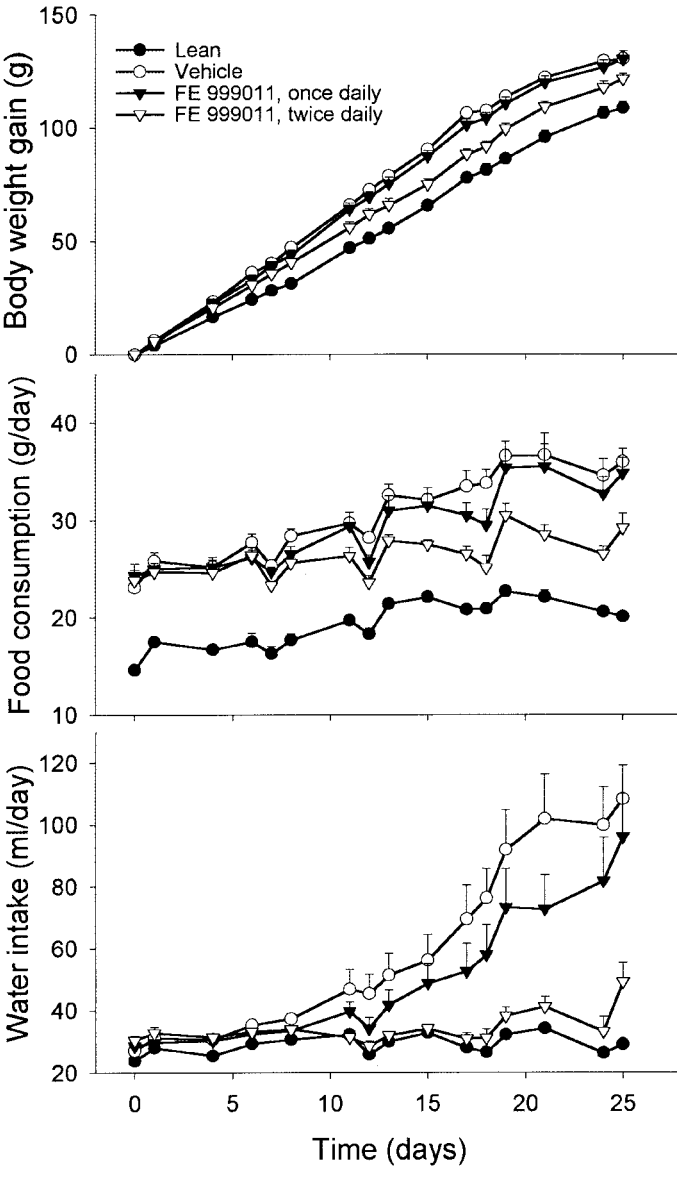

FIG. 7. Pattern of changes in body weight gain, food intake, and water intake of lean rats (●) and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day (▼) or twice a day (▽). Data are the means ± SE, n = 8 rats per group.

the experiment ($F_{63,447} = 22.8$, $P < 0.001$). The obese groups ate significantly more than the lean control group. Food consumption increased with time in the control obese group and the group treated once a day with FE 999011. From day 17 to the end of the treatment period, FE 999011 twice a day stabilized food intake to pretreatment level. Water intake in the four groups strikingly differed throughout the time course of the experiment ($F_{63,444} = 14.9$, $P < 0.001$). Ten days after the start of the experiment, water intake steadily increased in the control obese group and the group treated with FE 999011 once a day. FE 999011 twice a day fully prevented this large increase in water consumption.

Pattern of changes in insulinemia. The three obese groups had increased basal plasma insulin levels at the beginning of the experiment in comparison to lean controls (Fig. 8). Insulinemia in the control obese group gradually increased to a peak value of 23.3 ± 3.6 ng/ml 8

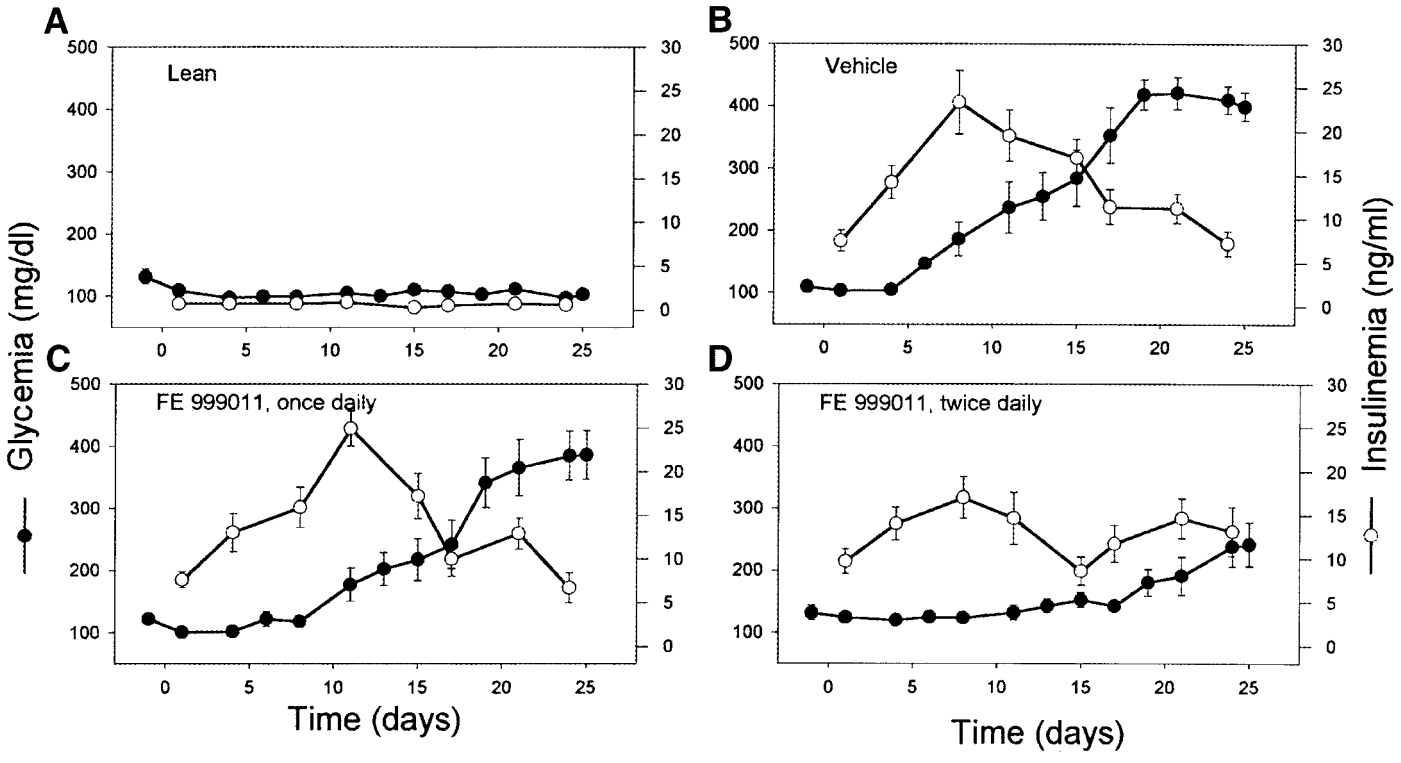

FIG. 8. Comparison of the pattern of changes in glycemia (●) and insulinemia (○) measured in lean control rats (*A*) and obese male ZDF rats receiving vehicle (*B*) or treated with 10 mg/kg FE 999011 once a day (*C*) or twice a day (*D*). Data are the means ± SE, $n = 8$ rats per group.

days after the initiation of treatment. Then, plasma insulin steadily decreased to a value of 7.2 ± 1.4 ng/ml at day 24 of the study. Obese rats treated with FE 999011 once a day showed a similar pattern of insulin secretion during this treatment period, with a peak of 24.9 ± 2 ng/ml at day 11 and a low value of 6.7 ± 1.7 ng/ml at day 24. The fall in insulinemia observed in the vehicle and once-a-day treated rats clearly coincided with the appearance of overt diabetes characterized by escalating plasma glucose levels. In obese rats treated with FE 999011 twice a day, basal insulin levels were generally lower and exhibited a biphasic pattern that differed from the two other obese groups. A first peak of insulinemia was seen at day 8 (17 ± 2.4 ng/ml), a trough after 15 days, and a second peak (14.6 ± 2.3 ng/ml) 21 days after initiation of treatment.

Plasma GLP-1 levels. Basal plasma levels for GLP-1 were measured in the fed state 25 days after initiation of treatment. Mean plasma level was lowest in untreated, obese diabetic rats (4.0 ± 0.1 pmol/l). Chronic treatment with FE 999011 increased basal GLP-1 plasma concentration to 5.8 ± 0.5 pmol/l in once-a-day treated rats and 6.2 ± 0.5 pmol/l in twice-a-day treated rats, with both increases being significant versus vehicle-treated obese rats ($F_{3,27} = 3.649$, $P < 0.025$). The value for lean rats was 5.2 ± 0.7 pmol/l.

Plasma DPP-IV activity. At the end of the study (day 26), plasma DPP-IV activity was 0.79 ± 0.03 in lean and 1.02 ± 0.05 rate/min in untreated obese rats, whereas a value of 0.23 ± 0.02 rate/min was measured in obese rats injected with FE 999011 twice a day ($F_{2,21} = 105.8$, $P < 0.001$).

Plasma FFA and TG levels. Plasma FFA and TG levels in the three obese groups were significantly higher than in lean rats (Fig. 9 and Table 1). Throughout the study, in vehicle-treated rats, there was a large increase in plasma levels of both FFAs and TG, whereas in the lean group, no change in plasma FFAs and a small increase in TGs were observed ($F_{15,112} = 18.5$, $P < 0.001$; $F_{16,119} = 42.4$, $P < 0.001$ for FFAs and TGs, respectively). Interestingly, mean plasma FFA levels did not increase significantly with the twice-a-day treatment (1.52 ± 0.16 to 1.84 ± 0.18 mEq/l), thus remaining in the concentration range seen in the prediabetic state (1.81 ± 0.17 mEq/l). Treatment with FE

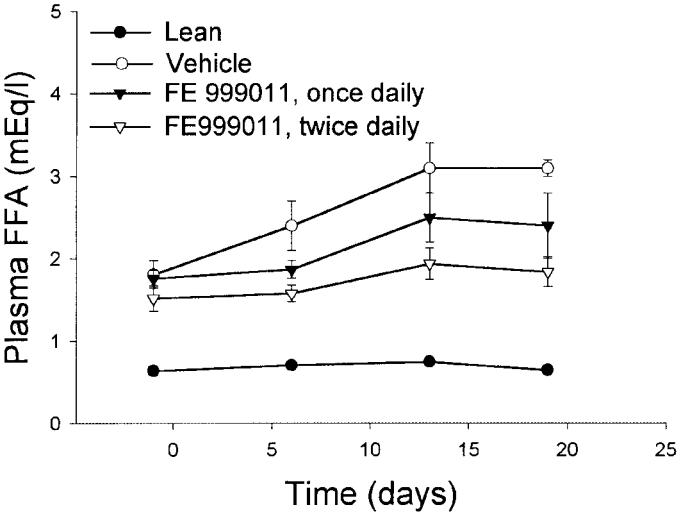

FIG. 9. Pattern of changes in plasma FFAs in lean rats (●) and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day (▼) or twice a day (▽). Data are the means ± SE, $n = 8$ rats per group.

TABLE 1
Plasma FFA and TG levels in ZDF rats

	Plasma FFA (mEq/l)		Plasma TG (ng/ml)	
	Day −1	Day 19	Day −1	Day 19
Lean FA/?				
No treatment	0.64 ± 0.04	0.65 ± 0.04	61.6 ± 4.1	94.5 ± 4.6
Obese *fa/fa*				
Vehicle	1.81 ± 0.17*	3.10 ± 0.1*	348.6 ± 16.7*	1254.5 ± 45*
FE 999011 once a day	1.76 ± 0.1*	2.40 ± 0.4*†	342.2 ± 13.6*	993.6 ± 130.9*†
FE 999011 twice a day	1.52 ± 0.16*	1.84 ± 0.18*†	327.3 ± 39.7*	672.4 ± 69*†‡

*$P < 0.05$ vs. lean; †$P < 0.05$ vs. obese vehicle; ‡$P < 0.05$ vs. obese FE 999011 once daily.

999011 significantly and dose-dependently attenuated the increase in TG values in the obese rats.

Gene expression for GLP-1 receptor in the pancreas. Gene expression for GLP-1R (as characterized by the number of mRNA copies per microgram of extracted total RNA from the whole pancreas) was increased in obese compared with lean control rats (Fig. 10). Treatment of obese rats with FE 999011 increased gene expression from $4.9 \pm 1.0 \times 10^6$ to $7.8 \pm 1.2 \times 10^6$ mRNA copies/μg total RNA in the once-a-day treated obese rat group (NS) and to 14.4 ± 3.2 in the twice-a-day treated group, a significant increase over the untreated group ($F_{3,26} = 49.0, P < 0.001$).

DISCUSSION

The new DPP-IV inhibitor FE 999011 described in this article was able to inhibit circulating DPP-IV activity and therefore improve GLP-1 secretion after a glucose load; it then effectively reduced the glucose excursion following OGTT in Zucker fatty rats to values in lean controls.

One major advantage of FE 999011 resides in its long duration of action, which extends ~12 h, so that twice-a-day oral administration continuously inhibits DPP-IV activity. Such chronic administration of FE 999011 to Zucker fatty rats led to a robust improvement in glucose tolerance: after 7 days of twice-a-day oral administration, the glucose excursion following OGTT was normalized even in absence of inhibitor given at the time of the glucose load. The fact that such improved glucose tolerance occurred with a reduced insulin surge suggests a significant improvement in insulin sensitivity. In the male ZDF rat, chronic treatment with FE 999011 significantly delayed the rise in glycemia. Our data clearly indicate that prevention of the diabetic situation was possible only with the twice-a-day administration of FE 999011, suggesting that continuous inhibition of circulating DPP-IV activity is required for optimal efficacy. With the once-a-day treatment, only a short-lived delay in onset of hyperglycemia was observed, and the pattern of changes in insulin secretion, food consumption, water intake, and body weight gain were not modified compared with untreated ZDF rats. With the twice-a-day treatment, euglycemia was maintained for at least 21 days with low basal insulin secretion, suggesting improved insulin sensitivity. Normal control of water intake, a hallmark of adequate control of glucose metabolism in diabetic rats (27), was another sign of treatment efficacy. During the last 10 days of treatment, both basal insulin and glucose levels started to rise moderately.

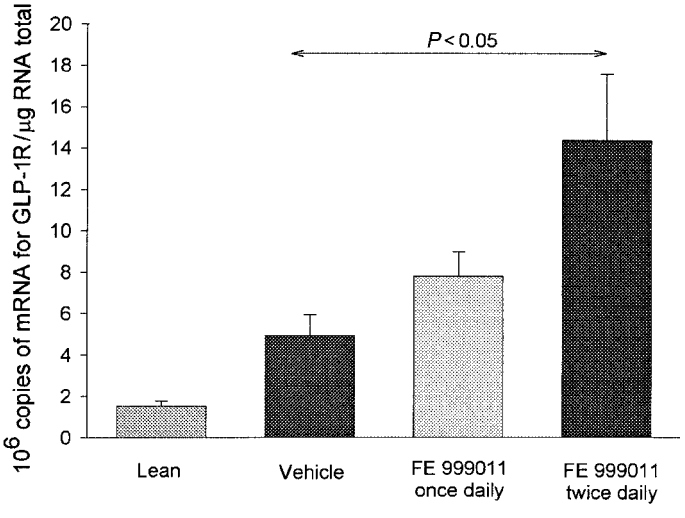

FIG. 10. Pattern of changes in pancreatic gene expression for GLP-1 receptor (GLP-1R), as quantified by RT-PCR. Data are the means ± SE, $n = 8$ rats per group.

One key outcome in this study was the improvement in glucose tolerance when circulating DPP-IV activity was chronically inhibited. This was clearly observed in both Zucker fatty and ZDF rats. It can be suggested that improvements of both glucose-induced insulin secretion and insulin sensitivity were determining factors for the prevention of diabetes in male ZDF rats. Stabilization of circulating FFAs to pretreatment levels obtained with the twice-a-day treatment was probably another determining factor for the maintenance of islet cell function in this model. The improvement of insulin secretion and efficacy observed in this study could have been mediated by enhanced GLP-1 secretion. Enhanced GLP-1 action at the level of rat pancreatic islets using GLP-1 analog infusion can increase insulin release (28). Plasma GLP-1 response upon an intraduodenal glucose load was greatly improved in FE999011-treated Zucker rats, and basal plasma GLP-1 levels were significantly increased by FE 999011, 25 days after initiation of treatment of ZDF rats.

GLP-1R is expressed in pancreatic islets and hypothalamus (29,30). In our study, gene expression for GLP-1R in the whole pancreas was found to be significantly increased in untreated obese compared with lean animals and was further upregulated by the twice-a-day FE 999011 treatment. The latter observation could suggest an increased sensitivity to the action of GLP-1, consistent with the improvement in insulin secretion and action. Because gene expression analysis was performed in total RNA extracted from the whole pancreas, GLP-1R mRNAs present in all types of islet cells (31,32) were detected.

Therefore, such an increase could have happened on each islet cell type, apparently improving each GLP-1 action at the pancreatic level. Another word of caution relates to whether increased gene expression automatically means increased cell sensitivity to the ligand. Regulation of GLP-1 action also depends on postreceptor signaling pathways. In the case of GLP-1, it has been shown in vitro that overexpression of GLP-1R can lead to desensitization (33). In view of the marked improvement in control of glucose homeostasis in this rat model following stabilization of GLP-1 action, one can hypothesize that an increased pancreatic GLP-1R population can be considered a positive factor. However, the mechanism and meaning of such change clearly require further evaluation.

GLP-1 is also known to reduce feeding through a brain-originating peptide acting on hypothalamic GLP-1 receptors (34,35). In this study, a hypothalamic anorectic effect of GLP-1 is very unlikely as a primary cause for the prevention of diabetes by FE 999011, as no anorectic effect was observed in Zucker rats after 7 days of treatment or at any time in ZDF rats chronically treated with FE 999011 twice a day. The role of circulating FFAs and TGs in the occurrence of the diabetic syndrome in leptin-resistant ZDF rats has been well discussed in the literature (36,37). In particular, the unusual accumulation of TGs in islet cells due to the absence of leptin action has been postulated to represent a major cause of failure of β-cells to produce and release insulin (38). Nonadipose tissues of normal rodents have a low TG content, even when fed a high-fat diet. In contrast, in rodents with a loss-of-function mutation in the leptin receptor, the TG content of certain nonadipose tissues is markedly increased, even on a normal fat intake (39). In ZDF rats, a 10- to 50-fold increase in TG content of the pancreatic islets has been reported (39). This is associated with impairment of β-cell function and ultimate loss of β-cells through lipoapoptosis, which results in diabetes (39,40). Thus lack of leptin action at the level of the β-cells in the ZDF rat and the resulting increase in pancreatic TG content could be the primary cause for loss of glucose-induced insulin secretion. During the course of our study, plasma levels for FFAs and TGs in the obese ZDF rat increased by two- and fourfold, respectively. The increase in plasma FFAs was prevented by the twice-a-day treatment with FE 999011 and maintained below the 2 mEq/l level that is toxic for β-cells (39,41). With the once-a-day treatment, prevention of this increase in plasma FFAs was less efficient. With the prevention of overt diabetes thanks to adequate insulin secretion and improvement of insulin sensitivity, insulin action on lipid metabolism is also enhanced and leads to a maintenance of moderate circulating FFA and TG levels. A combination of decrease in FFA and TG levels and enhanced GLP-1 action, both leading to preservation of β-cell function, represents the likely mechanism for the successful prevention of diabetes by FE 999011.

This study demonstrated that chronic inhibition of DPP-IV by FE 999011 can delay the occurrence of type 2 diabetes in ZDF rats. Permanent inhibition of circulating DPP-IV activity most likely leads to a sustained action of GLP-1 and possibly GIP at the pancreatic level. GLP-1 also stimulates increases in β-cell mass and insulin synthesis and thus favors adequate insulin stores in pancreatic islets. Furthermore, GLP-1 can enhance insulin action by inhibiting glucagon secretion (42). It is therefore speculated that in the clinic, DPP-IV inhibitors such as FE 999011 could delay or even prevent the progression from impaired glucose tolerance to type 2 diabetes by improving glucose tolerance and preserving β-cell function. Of importance for a DPP-IV–based treatment is assessment of its effect on the immune response (43,44) and also determination of whether other endocrine axes are modified, since inhibition of DPP-IV activity may enhance the action of other polypeptide hormones that are susceptible to cleavage and inactivation, such as neuropeptide Y (45) or growth hormone–releasing hormone (46).

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