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GLP-1 derivative liraglutide in rats with β-cell deficiencies: influence of metabolic state on β-cell mass dynamics

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1 Liraglutide is a long-acting GLP-1 derivative, designed for once daily administration in type II diabetic patients. To investigate the effects of liraglutide on glycemic control and β-cell mass in rat models of β-cell deficiencies, studies were performed in male Zucker diabetic fatty (ZDF) rats and in 60% pancreatectomized rats.

2 When liraglutide was dosed s.c. at 150 $\mu g\,kg^{-1}$ b.i.d. for 6 weeks in ZDF rats 6–8 weeks of age at study start, diabetes development was markedly attenuated. Blood glucose was approximately 12 mM lower compared to vehicle ($P<0.0002$), and plasma insulin was 2–3-fold higher during a normal 24-h feeding period ($P<0.001$). Judged by pair feeding, approximately 53% of the antihyperglycemic effect observed on 24-h glucose profiles was mediated by a reduction in food intake, which persisted throughout the study and averaged 16% ($P<0.02$).

3 Histological analyses revealed that β-cell mass and proliferation were significantly lower in prediabetic animals still normoglycemic after 2 weeks treatment compared to vehicle-treated animals that had begun to develop diabetes. When the treatment period was 6 weeks, the liraglutide-treated animals were no longer completely normoglycemic and the β-cell mass was significantly increased compared to overtly diabetic vehicle-treated animals, while β-cell proliferation was unaffected.

4 In the experiments with 60% pancreatectomized rats, 8 days treatment with liraglutide resulted in a significantly lower glucose excursion in response to oral glucose compared to vehicle treatment. Again, part of the antihyperglycemic effect was due to reduced food intake. No effect of liraglutide on β-cell mass was observed in these virtually normoglycemic animals.

5 In conclusion, treatment with liraglutide has marked antihyperglycemic effects in rodent models of β-cell deficiencies, and the *in vivo* effect of liraglutide on β-cell mass may in part depend on the metabolic state of the animals.
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Abbreviations: AUC, area under the curve; b.i.d., twice a day; BrdU, bromodeoxyuridine; DPPIV, dipeptidyl-peptidase IV; GLP-1, glucagon-like peptide-1; OGTT, oral glucose tolerance test; ZDF, Zucker diabetic fatty

Introduction

Glucagon-like peptide-1 (GLP-1) is secreted from the intestinal L-cells in response to ingested carbohydrates and fat (Kreymann *et al*., 1987; Ørskov, 1992; Holst, 1997). The molecule has a spectrum of physiological effects, including glucose-dependent stimulation of insulin secretion and inhibition of glucagon secretion (Gromada *et al*., 1998), inhibition of small bowel motility (Tolessa *et al*., 1998) and gastric emptying (Nauck *et al*., 1997), and reduction of appetite (Flint *et al*., 1998; Toft-Nielsen *et al*., 1999). Moreover, recent evidence suggests that GLP-1 and analogs act as trophic agents in the pancreas, causing β-cell proliferation and neogenesis (Buteau *et al*., 1999; Edvell & Lindström, 1999; Xu *et al*., 1999; Perfetti *et al*., 2000; Moldrup *et al*., 2001; Farilla *et al*., 2002; Rolin *et al*., 2002), and some studies indicate that it also inhibits β-cell apoptosis (Bregenholt *et al*., 2001; Farilla *et al*., 2002; Li *et al*., 2003). Thus, GLP-1 may be an important physiological regulator of β-cell mass. Taken as a whole, these properties make GLP-1 ideally suited to be developed as an antidiabetic agent, particularly since many of its effects are glucose-dependent and therefore treatment would likely be associated with a very low risk of hypoglycemia. Consequently, many studies have been undertaken and shown GLP-1 to be highly effective in reducing blood glucose levels in patients with type II diabetes (Nauck *et al*., 1993; Gutniak *et al*., 1994; Rachman *et al*., 1997; Larsen *et al*., 2001a). Native GLP-1 is, however, rapidly degraded by dipeptidyl peptidase IV (DPPIV; Mentlein *et al*., 1993; Deacon *et al*., 1995) and cleared by the kidneys (Deacon *et al*., 1996), giving the biologically active peptide a half-life of less than 2 min after i.v. administration (Deacon *et al*., 1996) and 1–2 h after s.c. administration (Knudsen *et al*., 2000). Since the presence of an elevated GLP-1 concentration is necessary in type II diabetic patients in order to obtain a continuous effect, the pharmacokinetic properties of the native

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peptide make it less than ideal for therapeutic use. Liraglutide (Arg^{34}Lys^{26}-(*N*-ε-(γ-Glu(*N*-α-hexadecanoyl)))-GLP-1(7-37)), also known as NN2211, is a novel long-acting analog, obtained by derivatizing GLP-1 with a fatty acid side chain, which promotes albumin binding and prevents degradation by DPPIV (unpublished observations). Liraglutide and GLP-1 are equipotent *in vitro* (Knudsen *et al*., 2000). When injected s.c., these features, combined with a slow release from the injection site, result in a compound with a prolonged plasma half-life of 14 h in pigs (Knudsen *et al*., 2000) and 10–12 h in man (Agersø *et al*., 2002; Juhl *et al*., 2002), which makes it suitable for once daily administration (Knudsen *et al*., 2000).

The present study was undertaken to investigate the effects of liraglutide on glycemia and β-cell mass in two β-cell-deficient rat models: male Zucker diabetic fatty (ZDF) rats, a model of type II diabetes in which insulin resistance and β-cell defects are prominent features (Clark *et al*., 1983; Etgen & Oldham, 2000), and 60% pancreatectomized Sprague–Dawley rats, a model of β-cell deficiency (Liu *et al*., 2000).

Methods

Animals

All studies were carried out with permits from the Animal Experiments Inspectorate, Ministry of Justice, Denmark. Male ZDF (*fa*/*fa*) rats (ZDF) and lean male ZDF (*fa*/+ or +/+) rats (lean) were purchased from Genetic Models Inc. (Indianapolis, IN, U.S.A.) and housed two per cage (study 1a and 1c) or individually (study 1b). Male Sprague–Dawley rats were obtained from M&B, Lille Skensved, Denmark, and were housed individually. Unless otherwise stated, animals had free access to food (Purina 5008) and drinking water.

Experimental procedures

Dosing In all studies, the animals were dosed with subcutaneous injections (1 ml kg^{-1}) of vehicle or liraglutide at the various doses indicated at approximately 0715–0800 and 1400–1415 h. Twice daily dosing was used because the pharmacokinetic half-life of liraglutide is only approximately 4 h in rats (Novo Nordisk, data on file).

Blood sampling Blood for the determination of whole-blood glucose concentration and HbA_{1c} was collected into heparinized 10 and 5 μl glass tubes, respectively, by puncture of the capillary vessels in the tail tip, and diluted in analysis buffer. Samples for insulin measurements were also collected from the tail into 100-μl heparin-coated glass tubes and were centrifuged at 4°C for 10 min and plasma was separated and stored at −20°C until assayed.

Oral glucose tolerance test Oral glucose tolerance tests (OGTTs) were performed in fasted animals. For ZDF rats, 1 g kg^{-1} glucose solution and for 60% pancreatectomized rats, 2 g kg^{-1} glucose solution was administered by gavage. Compound or vehicle was administered at the regular time points. Samples for the measurement of blood glucose and, in some cases, plasma insulin were drawn immediately prior to glucose administration and at intervals thereafter.

Studies in ZDF rats

These studies were performed to investigate the ability of liraglutide to influence diabetes development and β-cell proliferation and mass in the ZDF rat model.

Study 1a Three groups of animals ($n=6$; age 7–8 weeks at the start of the study) were housed two per cage and treated b.i.d. with either vehicle or liraglutide (low dose, 30 μg kg^{-1}; high dose, 150 μg kg^{-1}) for 6 weeks. Initial random glucose concentrations were not significantly different between the three groups (vehicle: 6.55±0.33; low dose: 6.40±0.22; high dose: 6.42±0.20 mM). Blood samples for 24-h profiles of glucose were taken approximately every 4 h on day 9, OGTTs were performed on days 21 and 36, and a final 24-h profile of glucose and insulin was carried out on day 41. Food and water intake were monitored daily (at approx. 0800 h), and body weight was measured regularly.

Study 1b Pair feeding was employed in order to investigate to what extent the blood glucose-lowering effect observed with 150 μg kg^{-1} liraglutide in study 1a could be ascribed to the reduction in food intake. Four groups of animals (age 6 weeks at the start of the study) were individually housed and treated b.i.d. with either vehicle (lean, $n=9$; ZDF vehicle, $n=7$; ZDF pair-fed, $n=8$) or liraglutide (150 μg kg^{-1}, $n=8$) for 6 weeks. Pair-matching was based on the animals' initial body weights. Daily food consumption was measured for each liraglutide-treated animal and this amount of food was then made available to the pair-matched vehicle-treated animal on the following day. Glucose and insulin profiles (24 h) were assessed on day 38. Food and water intake were measured daily and body weight was recorded twice weekly.

Study 1c Two groups of animals ($n=9$–10; age 8 weeks at the start of the study) received either vehicle or liraglutide (200 μg kg^{-1}) b.i.d. for 2 weeks. OGTTs were performed after the first dose and again after 13 days. Food and water consumption were recorded throughout the study period. Body weight and fructosamine were measured before and at the end of the study.

Studies in 60% pancreatectomized rats

These studies were performed to test the effect of short-term liraglutide treatment on glucose tolerance and β-cell mass in 60% pancreatectomized rats, a nongenetic model of β-cell deficiency.

Study 2a In all, 12 male Sprague–Dawley rats of 80–100 g received presurgical streptocillin treatment (50 μl (100 g^{-1}) i.m.) prior to isofluoran anesthesia and were subjected to 60% pancreatectomy using the method previously described (Liu *et al*., 2000). Briefly, the portion of the pancreas bordered by the spleen and ventriculus extending to the duodenum was carefully removed by gentle abrasion with cotton applicators, leaving the ligament with arteries and veins intact. The remnant pancreatic tissue was anatomically defined as the duodenal segment. All animals received postsurgical pain relief Finadyne (20 μl (100 g^{-1}) i.m.) and were allowed to recuperate for 4 days postoperatively. Animals were allocated into two groups ($n=6$), based on an OGTT on day 4, after which they

received either vehicle or liraglutide (100 $\mu g\,kg^{-1}$) b.i.d. for 4 days. A second OGTT was carried out on day 8.

Study 2b In all, 24 male Sprague–Dawley rats were subjected to 60% pancreatectomy and allocated into three groups ($n=8$) after an OGTT on day 4. One group was thereafter treated with vehicle, a second vehicle-treated group was pair-fed relative to the liraglutide-treated group, and the third group received liraglutide (150 $\mu g\,kg^{-1}$) b.i.d., all for 4 days. A second OGTT was carried out on day 8.

Histology In all studies, 4 h prior to killing, animals were injected with bromodeoxyuridine (BrdU, 100 $mg\,kg^{-1}$ i.p.). The animals were anesthetized with CO_2, decapitated, and approximately 5 ml whole blood for determination of fructosamine (study 1a) and liraglutide concentrations (study 1b) was collected from the trunk into 10 ml tubes containing 0.12 ml 15% K_3 ETDA. The pancreata were immediately isolated for histological analysis, weighed, fixed in 4% paraformaldehyde overnight, cut in small pieces, fractionated according to the smooth fractionator principle (Bock *et al*., 1999), dehydrated, and embedded in paraffin. Paraffin sections (3 μm) were cut, deparaffinized, rehydrated, the endogenous peroxidase blocked by H_2O_2 in 96% ethanol, and blocked with avidin and then biotin, before use for immunohistochemical staining of islet cell markers, which was carried out with slides from all pancreata in parallel. For antigen retrieval, the sections in 0.01 M citrate buffer pH 6.0, preheated to 90°C, were heated 3×5 min in a microwave oven, before blocking with avidin and biotin blocking solutions (DAKO) and 10% normal goat serum. BrdU was stained by monoclonal mouse anti-BrdU (M0744, DAKO) 1 : 50 in 7% goat serum 3% rat serum in TBS, biotinylated goat anti-mouse Ig (E0433, DAKO) 1 : 300, and streptavidin peroxidase (Vectastain, Vector). The peroxidase activity was developed 5 min with 0.066% diaminobenzidine (DAB) + 0.01% H_2O_2 + 2.5% $NiSO_4$ to render BrdU containing nuclei black. Insulin was stained with guinea-pig antiinsulin (#651041, ICN) 1 : 400 in 7% rabbit serum 3% rat serum in TBS-T, peroxidase-coupled rabbit anti-guinea-pig Ig (P141, DAKO) 1 : 100, and developed with DAB for brown cytoplasm or with Vector Nova Red (Vector) according to the manufacturer, to stain the β-cell cytoplasm reddish brown. Finally, the slides were lightly counterstained with Mayer's hematoxylin. Neighboring sections were stained for non-β-cells with a mixture of monoclonal mouse antiglucagon (GLU-001, Novo Nordisk) 1 : 800 + rabbit antisomatostatin (A566, DAKO) 1 : 600 + rabbit antipancreatic polypeptide (A619, DAKO) 1 : 1000 in 4% swine serum + 4% goat serum + 3% rat serum in TBS-T, detected using biotinylated goat anti-mouse IgG (E0433, DAKO) 1 : 400 + biotinylated swine anti-rabbit (E353, DAKO) 1 : 400, streptavidin peroxidase, and developed with DAB and $NiSO_4$, as above. In ZDF studies 1a and 1b, β-cells were subsequently stained for insulin as described above. Reagents were obtained from DAKO, Copenhagen, Denmark, if not otherwise stated. BrdU staining of cell nuclei was examined in 1000–1500 β-cells per section in an Olympus BX-50 microscope with a video camera and monitor (Olympus, Copenhagen, Denmark), at a total on-screen magnification of $\times1920$. The sections were systematically scanned using a PC-controlled motorized stage and CAST-GRID software (Olympus). The volume fractions of β- and non-β-cells were estimated by point-counting stereological techniques, at a total on-screen magnification of $\times960$, a grid of 6×64 points, and step lengths of $1200\times1000\,mm^2$. The sections were examined with their origin blinded to the observer. Two sections cut 250 μm apart were stained and analyzed for each set of estimations.

Assays Glucose concentrations were measured by the immobilized glucose oxidase method using an EBIO Plus autoanalyser (Eppendorf, Germany). Plasma insulin concentration was measured with an in-house ELISA method (Johansen *et al*., 1999) using 15 μl samples. Rat insulin was used as standard. Per cent HbA_{1c} (Roche A/S) was measured by the enzymatic calorimetric method (COBAS MIRA Plus Autoanalyser, Roche Diagnostic Systems, Basel, Switzerland). The concentration of liraglutide was measured by sandwich ELISA (Wilken *et al*., 2000).

Statistics Paired and unpaired *t*-tests and one-way analysis of variance (ANOVA) with Duncan's and Tukey's *post hoc* tests for pairwise group comparisons were employed. Area under the curve (AUC) was calculated with the trapezoidal rule. Data are expressed as mean ± s.e.m. Differences with $P<0.05$ were considered significant.

Results

ZDF studies: metabolic parameters

Glucose tolerance tests *Study 1a*: Both the low and high doses of liraglutide significantly lowered glucose excursions (AUC) compared to vehicle treatment. Differences were seen both during the first OGTT (Figure 1; day 21; $P<0.0005$, ANOVA), and during the second OGTT (Figure 1; day 36; AUCs: vehicle 2250 ± 90 mM min vs low dose 1450 ± 180 mM min vs high dose 760 ± 40 mM min, $P<0.0002$, ANOVA, all groups different by *post hoc* testing), demonstrating a continuing antihyperglycemic effect at both dose levels. Plasma insulin concentrations were also increased in a dose-dependent manner (AUCs: vehicle 33 ± 5 nM min and low dose 59 ± 12 nM min vs high dose 117 ± 8 nM min, $P<0.0002$, ANOVA, high dose different from low dose and vehicle) and the effect of the drug increased with time. Thus, in the high-dose group, the AUC of insulin was increased between the first and second OGTT ($P<0.005$, paired *t*-test), the increase averaging 66%. In contrast, in the vehicle group, the AUC of insulin was decreased between the first and second OGTT ($P<0.0005$, paired *t*-test), the decrease averaging 40%. In the second OGTT, the effect on the second phase of insulin secretion appeared particularly pronounced in the high-dose group.

Study 1c: In the shorter-term study, no effect of the first dose was observed on glucose or insulin excursion during the OGTT, while significant effects on both parameters were observed on day 13 (data not shown).

Profiles (24 h), fructosamine and HbA1c *Study 1a*: After 41 days treatment, the final 24-h glucose profile (Figure 2) was significantly lower in the high-dose liraglutide group, the difference to the vehicle-treated group averaged ~12 mM (glucose AUCs: vehicle 31 ± 1 M min and low dose 26 ± 3 M min vs high dose 14 ± 2 M min, $P<0.0002$, ANOVA, high dose

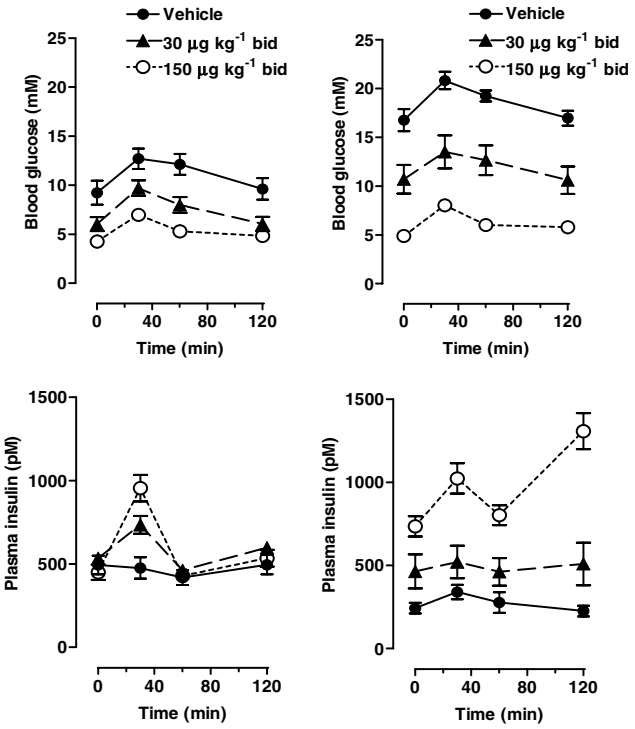

Figure 1 ZDF study 1a. OGTT after 21 days (left) and 36 days liraglutide treatment (right). Glucose (1 g kg^{-1}) was administered by gavage at time 0 to overnight fasted animals and BG and plasma insulin were measured basally and after the glucose challenge. All the three groups differed significantly with respect to total AUC after the glucose challenge ($P<0.0005$ by ANOVA, day 21, $P<0.0002$ by ANOVA, day 36).

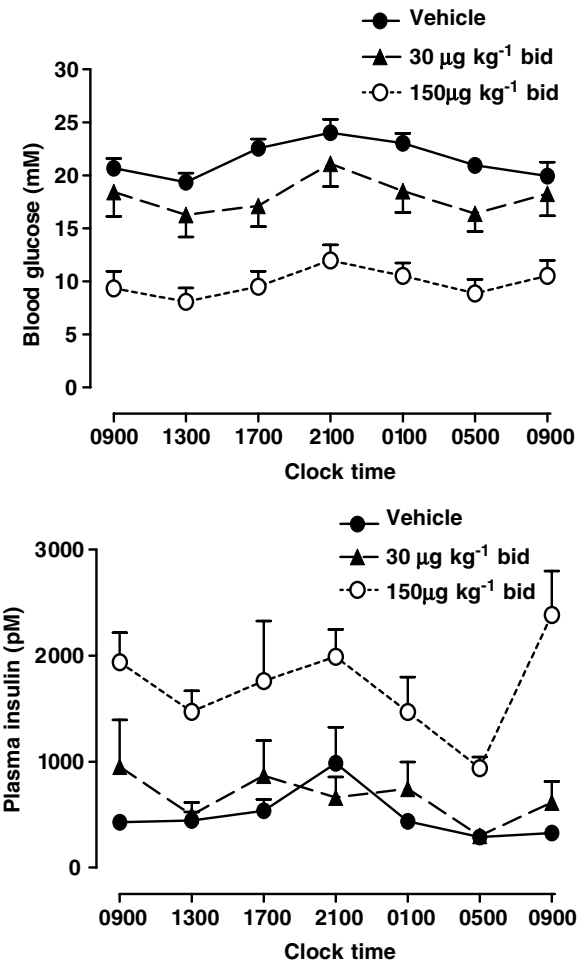

Figure 2 ZDF study 1a. Glucose and insulin profiles (24 h) after 41 days liraglutide treatment. Animals had free access to food throughout. Judged by 24-h AUCs, BG was significantly decreased ($P<0.0002$ by ANOVA) and plasma insulin was significantly increased ($P<0.002$ by ANOVA) in the group receiving high-dose liraglutide compared to low dose and vehicle.

different from low dose and vehicle). In contrast, the 24-h insulin profile demonstrated increased insulin levels in the high-dose group (insulin AUCs: vehicle 0.74 ± 0.12 μM min and low dose 0.92 ± 0.30 μM min vs high dose $2.35+0.34$ μM min, $P<0.002$, ANOVA, high dose different from low dose and vehicle). Fructosamine was significantly reduced in the high-dose group (vehicle 229 ± 7 μM and low dose 219 ± 11 μM vs high dose 174 ± 8 μM, $P<0.002$, ANOVA).

Study 1b: After treatment for 38 days, the AUCs of the 24-h glucose profiles were significantly different between all four groups (Figure 3). Lean animals had the lowest and ZDF vehicle animals the highest glucose AUC. Both liraglutide and pair feeding reduced the glucose AUC compared to the ZDF vehicle group. The reduction achieved by pair feeding alone was 7.5 M min, that is, approximately 53% of the 14.2 M min reduction observed with liraglutide treatment (Table 1). Similarly, HbA_{1c} was significantly reduced in both ZDF liraglutide and ZDF pair-fed animals compared to vehicle, but was not normal compared with lean vehicle (Table 1). AUC for plasma insulin did not differ between ZDF liraglutide and ZDF pair-fed, but both were higher than lean vehicle and ZDF vehicle, which were similar to each other (Table 1). The reproducibility of the glucose and insulin results compared with study 1a strongly suggests that any stress caused to the animals by the individual housing did not influence the development of diabetes.

Study 1c: Fructosamine levels were decreased in the liraglutide group compared to vehicle.

Food and water consumption, body weight

In all the three ZDF studies, liraglutide treatment had an immediate strong effect on lowering both food and water intake immediately after dosing was initiated. With continued dosing, a less pronounced, but significant effect on food and water consumption was observed. At the end of the study, the overtly hyperglycemic vehicle-treated ZDF rats consumed significantly more water than the remaining groups. The data from study 1b are representative and are summarized in Table 2. Hematocrit measured at the end of study 1a was not reduced by liraglutide treatment (data not shown), indicating that the reduced water consumption was not associated with dehydration. Within each of the three ZDF studies, initial body weight did not differ between ZDF groups and in the early treatment period, the reduced food intake in liraglutide-treated animals resulted in an attenuated weight gain. However, as the treatment duration increased, the difference in weight disappeared, reflecting a treatment-related reduced loss of calories due to glucosuria. Data on body weight from study 1b are representative and are illustrated in Figure 4.

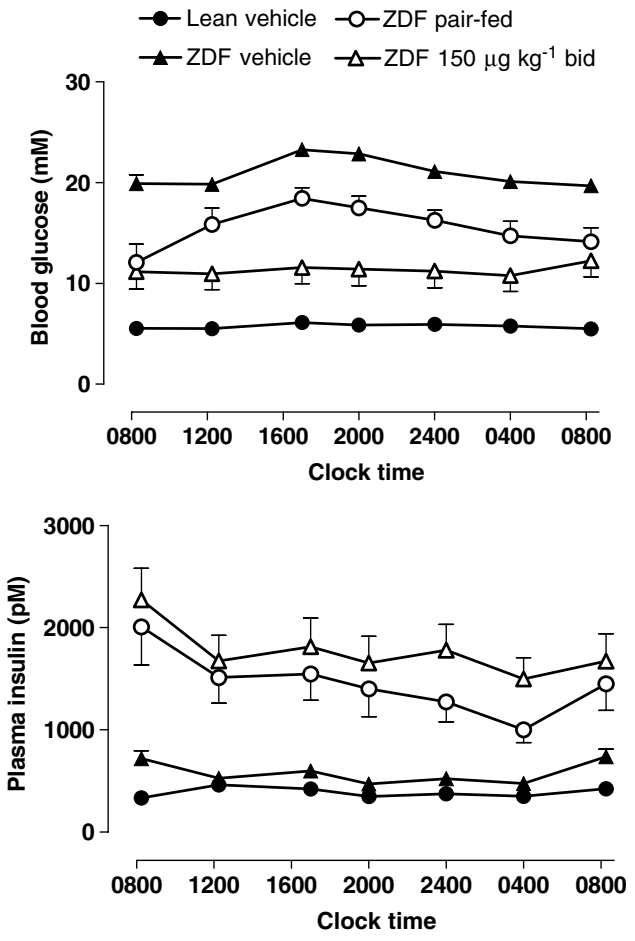

Figure 3 ZDF study 1b. Glucose and insulin profiles (24 h) after 38 days liraglutide treatment. Animals had free access to food throughout. Judged by 24-h AUCs, BG was significantly decreased ($P<0.0001$ by ANOVA) and plasma insulin was significantly increased ($P<0.0001$ by ANOVA) in animals receiving liraglutide compared to vehicle (see Table 1 for detailed analysis).

Table 1 ZDF, study 1b: AUC of glucose and insulin during 24-h profile and HbA_{1c} after 38 days treatment

	AUC glucose (M min)	*AUC insulin* (μM min)	*HbA_{1c}* (%)
Lean vehicle	8.3±0.1	0.6±0.02	3.9±0.04
ZDF vehicle	30.4±0.6*	0.8±0.1	8.6±0.2*
ZDF pair-fed	22.9±0.2*†	2.0±0.3*†	6.3±0.4*‡
ZDF liraglutide	16.2±0.2‡§‖	2.5±0.4*‡	5.5±0.4‡§
ANOVA	$P<0.0001$	$P<0.0001$	$P<0.0001$

*$P<0.001$ vs lean vehicle; †$P<0.01$ vs ZDF vehicle; ‡$P<0.001$ vs ZDF vehicle; §$P<0.01$ vs lean vehicle; ‖$P<0.05$ vs ZDF pair-fed, Tukey's *post hoc* test. Data are mean±s.e.m.

Plasma concentration liraglutide

In study 1b, the total plasma concentration (albumin bound + free) of liraglutide was 160±12 nM (approximately 5–7 h after the final dose). The high total concentration reflects the albumin-binding properties of liraglutide.

Table 2 ZDF, study 1b: food and water intake

	Average daily food intake (g)	*Average daily food intake, final week* (g)	*Average daily water intake, final week* (g)
Lean vehicle	20.2±0.4	20.0±0.6	29±1#
ZDF vehicle	30.4±0.7*	30.8±0.9*	94±11
ZDF pair-fed	23.2±0.3*#	23.5±0.5§#	56±4§#
ZDF liraglutide	23.8±0.4*#	25.1±0.6*#	38±3#
ANOVA	$P<0.0001$	$P<0.0001$	$P<0.0001$

*$P<0.001$ vs lean vehicle; #$P<0.001$ vs ZDF vehicle; §$P<0.01$ vs lean vehicle. Data are mean±s.e.m.

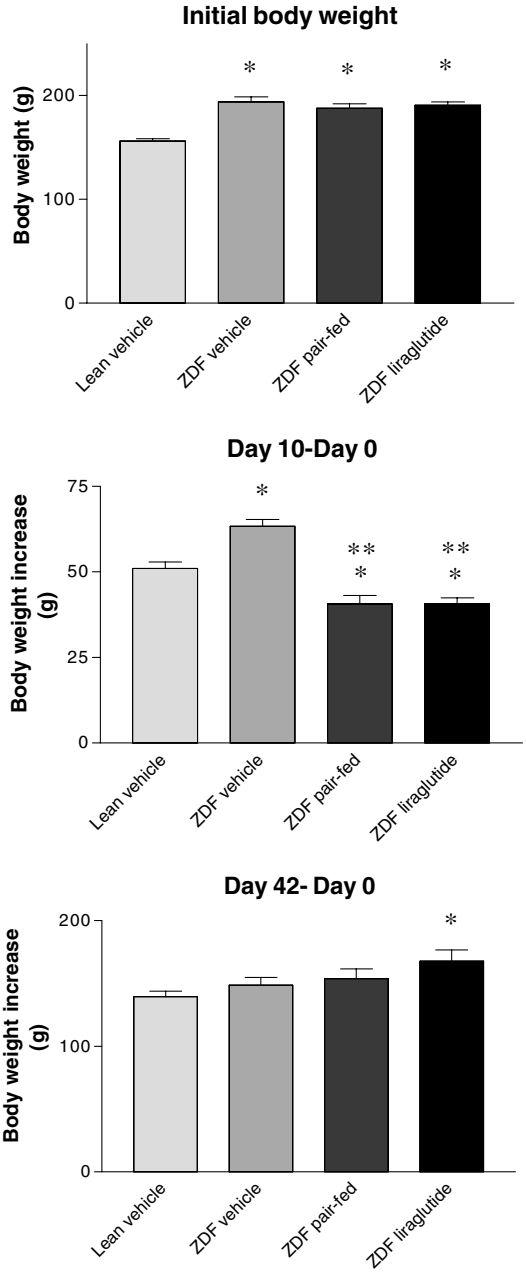

Figure 4 ZDF study 1b. Initial body weight and body weight increase after 10 and 42 days liraglutide treatment. *$P<0.01$ vs lean vehicle; **$P<0.001$ vs ZDF vehicle.

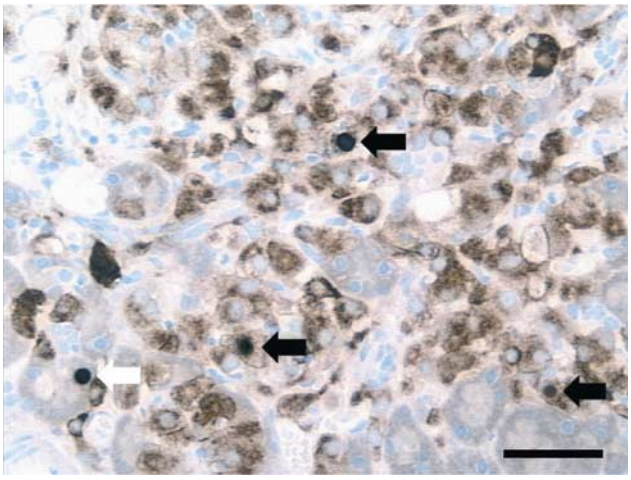

Figure 5 BrdU incorporation in β- and non-β-cells of the pancreas of a 13-week-old vehicle-treated diabetic ZDF rat from ZDF study 1a. Black nuclei = BrdU incorporation, brown cytoplasm = insulin in β-cells. Filled arrows show BrdU-positive β-cells, empty arrow shows a BrdU-positive non-β-cell (exocrine or centro-acinar duct cell?). Horizontal bar indicates 50 μm.

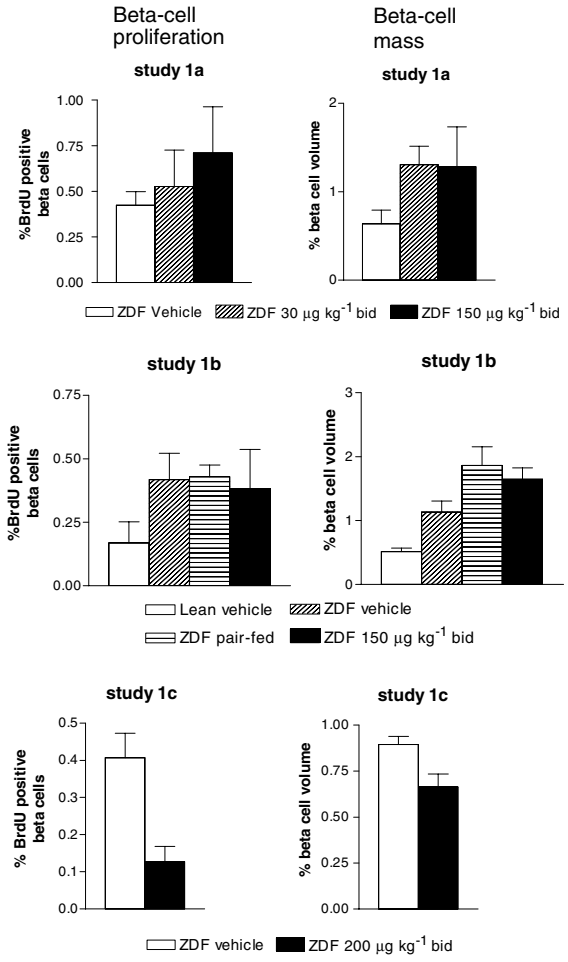

Figure 6 Quantitation of β-cell proliferation and mass (volume fraction) in ZDF experiments. Study 1a: after 6 weeks treatment, β-cell volume fraction was increased in both the low- and high-dose liraglutide groups, though only statistically significant for the low-dose group ($P<0.05$) There was a weak trend towards increased β-cell proliferation as estimated by the BrdU indices. Study 1b: β-cell volume fraction in liraglutide-treated and pair-fed groups were higher than in the lean control group, but not significantly higher than in the vehicle-treated ZDF rats. There was no difference between the β-cell proliferation rates of the three groups of ZDF rats. Study 1c: β-cell volume fraction was significantly lower in the animals treated with liraglutide ($P<0.002$), and this relative decrease occurred concomitantly with a markedly reduced β-cell proliferation ($P<0.006$).

ZDF studies: histology

Proliferation of β-cells was demonstrated by the incorporation of BrdU into β-cell nuclei (Figure 5). It should be noted that the number of non-β-cells in islets, duct cells, and exocrine cells that incorporated BrdU exceeded that of β-cells. The β-cell mass was estimated by point-counting morphometry. In study 1a, after 6 weeks treatment in the study, β-cell mass (volume fraction) was increased in both the low- and high-dose liraglutide groups, although only statistically significant for the low-dose group (Figure 6a), and there was a weak trend towards increased β-cell proliferation as estimated by the BrdU indices. In general, the islet morphology in all groups of ZDF rats was quite irregular and the β-cell insulin-staining intensity variable with no treatment-related differences in staining intensity (not shown). In study 1b, β-cell volume fractions in liraglutide-treated and pair-fed groups were higher than in the lean control group, but not higher than in the vehicle-treated ZDF rats (Figure 6b). When expressed in terms of total β-cell mass, the liraglutide-treated group of ZDF rats had a higher β-cell mass than the ZDF vehicle animals (20.9 ± 2.6 vs 12.4 ± 1.8 mg, $P<0.03$). There was no difference between the β-cell proliferation rates of the three groups of ZDF rats and no obvious differences between insulin-staining intensities.

After only 2 weeks treatment in study 1c, β-cell mass (volume fraction) was significantly lower in the animals treated with liraglutide (0.66 ± 0.04 vs $0.90\pm0.06\%$, $P<0.002$), and this relative decrease occurred concomitantly with a markedly reduced β-cell proliferation (0.13 ± 0.04 vs $0.46\pm0.07\%$, $P<0.006$, Figure 6c). In contrast, the insulin-staining intensity of β-cells in the liraglutide-treated rats was markedly higher than in the vehicle group (Figure 7).

Non-β-cells were demonstrated by a cocktail of antibodies against glucagon, somatostatin, and pancreatic polypeptide. Stereological measurements of the volume fractions of non-β-cells in the pancreata from these experiments showed no treatment-related differences between the liraglutide-treated groups of ZDF rats and the vehicle- (study 1a and 1b) or vehicle plus pair feeding (study 1b)-treated groups (not shown).

Studies in 60% pancreatectomized rats: metabolic parameters

In study 2a, liraglutide ($100\,\mu g\,kg^{-1}$ b.i.d.) treatment resulted in a tendency towards lower blood glucose during the OGTT compared to vehicle at day 8, but the difference in AUC for glucose was not statistically significant (Figure 8, top). However, in study 2b, the higher dose of liraglutide ($150\,\mu g\,kg^{-1}$ b.i.d.) resulted in significantly lower AUC for blood glucose at day 8 compared with both the vehicle and

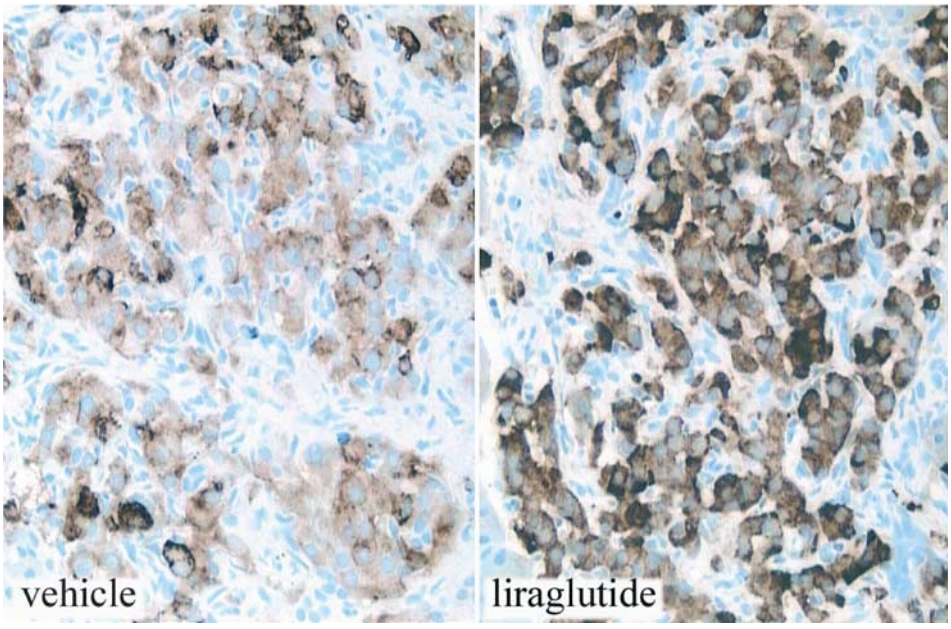

Figure 7 Increased insulin staining intensity after 2 weeks liraglutide treatment. Representative sections from pancreata of ZDF rats show higher and more regular insulin-staining intensity in the liraglutide-treated rat than in the vehicle-treated rat. Note the better staining pattern and intensity of this 10-week-old newly diabetic vehicle-treated rat than that of the 13-week-old diabetic rat from the experiment shown in Figure 5.

pair-fed groups. Furthermore, pair feeding itself resulted in slightly, but significantly, reduced delta AUC during the OGTT (Figure 8, bottom).

Studies in 60% pancreatectomized rats: histology

In both studies 2a and 2b, the distribution of islet sizes showed a trend towards an increase in the liraglutide-treated animals. However, point-counting morphometry at sections through the remnant pancreas from study 2b revealed no significant differences in the total β-cell volume fractions ($1.27 \pm 0.16\%$ in the liraglutide group, $1.15 \pm 0.15\%$ in the vehicle group, and $0.95 \pm 0.14\%$ in the pair-fed group).

There was no visible difference between the vehicle- and liraglutide-treated groups in the size or number of islets located in the regenerated area. Immunostaining for BrdU revealed a marked proliferation mainly in exocrine cells located at the border of the regeneration zones in both vehicle- and liraglutide-treated animals. At this time point, there was no effect of liraglutide treatment on the proliferative index in islets or in any other areas of remnant or regenerating pancreas.

Discussion and conclusions

We have demonstrated in two different rat models of β-cell deficiency that liraglutide has marked antihyperglycemic effects. In male ZDF rats, treatment with liraglutide strongly attenuated diabetes development in prediabetic animals, while treatment in 60% pancreatectomized rats reduced glucose excursions after an OGTT. In both models, the effects were partly, but not completely, mediated by a reduction in food intake. Furthermore, we have demonstrated that increases in β-cell proliferation and mass did not occur in situations when treatment was able to maintain normoglycemia.

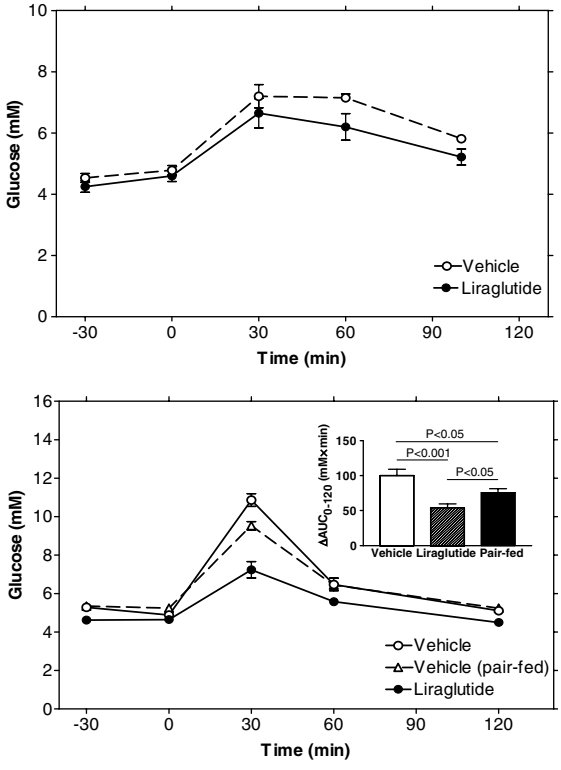

Figure 8 OGTTs in experiments performed in 60% pancreatectomized rats. After an overnight, fast glucose ($2\,g\,kg^{-1}$) was administered by gavage at time 0 to overnight fasted animals and BG was measured basally and after the glucose challenge. Top: study 2a. After dosing vehicle or $100\,\mu g\,kg^{-1}$ liraglutide b.i.d. for 4 days, a nonsignificant trend towards improved glucose tolerance was observed in the treated animals. Bottom: study 2b. After dosing vehicle or $150\,\mu g\,kg^{-1}$ liraglutide b.i.d. for 4 days, significantly lower AUC for BG was observed in both the vehicle and pair-fed groups. Furthermore, pair feeding itself resulted in slightly, but significantly, reduced delta AUC during the OGTT.

The male ZDF rat is a commonly used model of type II diabetes (Clark *et al*., 1983; Friedman *et al*., 1991; Sturis *et al*., 1995; Sreenan *et al*., 1996; Etgen & Oldham, 2000; Farilla *et al*., 2002). Homozygous males are severely insulin resistant compared to lean controls and have compensatory hyperinsulinemia at a young age (Etgen & Oldham, 2000). These features are caused by a mutation in the leptin receptor (Phillips *et al*., 1996) and, in this regard, the ZDF rat is like the Zucker *fa*/*fa* rat. Unlike the Zucker rat, however, the male ZDF rat develops frank diabetes, beginning between weeks 6 and 10 of age, when fed a diet containing 6.5% (weight%) fat (Purina 5008), and the animals simultaneously display a progressive deterioration of β-cell function (Etgen & Oldham, 2000). A genetic β-cell defect that segregates independently from the *fa* locus has recently been demonstrated in the ZDF rat (Griffen *et al*., 2001). Thus, it appears that two distinct genetic defects are responsible for diabetes in this model, one causing insulin resistance, and the other causing a progressive deterioration of β-cell function in the presence of insulin resistance, with the severity of disease influenced by dietary fat intake.

Exogenously administered GLP-1 stimulates insulin secretion and inhibits glucagon secretion, with both actions being glucose-dependent (Willms *et al*., 1996). Thus, at fasting glucose concentrations, GLP-1 causes little stimulation of insulin secretion, while at higher glucose concentrations, a much larger stimulation occurs. The signal transduction mechanism of GLP-1 on insulin secretion is not fully understood, but most likely involves cAMP generation and thus PKa activation, thereby potentiating the normal metabolic stimulus secretion mechanisms that regulate insulin secretion *via* the K_{ATP} channel (MacDonald *et al*., 2002). Liraglutide stimulates insulin secretion glucose-dependently in a pig model of glucose intolerance (Ribel *et al*., 2002), in keeping with the compound's mechanism of action as a full agonist at the GLP-1 receptor with an affinity equal to that of the native peptide (Knudsen *et al*., 2000). In addition to these pancreatic effects, exogenous GLP-1 inhibits gastric emptying dramatically (Wettergren *et al*., 1993; Willms *et al*., 1996; Nauck *et al*., 1997). The lack of an acute effect of liraglutide on insulin secretion in the present study is, thus, compatible with the animals' relatively low blood glucose levels, combined with a delay in gastric emptying. Indeed, it has been suggested that GLP-1's effect on gastric emptying is the major determinant of its glucose-lowering effects in healthy humans (Nauck *et al*., 1997). However, glucose tolerance tests in the ZDF animals showed that liraglutide treatment had a marked long-term effect, both on glucose tolerance and insulin secretion. Whereas vehicle-treated ZDF rats, as expected, started to become less hyperinsulinemic with the progressing disease, the liraglutide-treated animals exhibited an increase in insulin secretion over time. Since GLP-1 does not have a major direct insulin-sensitizing effect, at least acutely (Vella *et al*., 2000), the data suggest that liraglutide treatment leads to an improved ability of the β-cells to compensate for the insulin resistance in these animals. Recent evidence does suggest, however, that chronic (6 weeks) treatment with GLP-1 in type II diabetic patients can actually improve insulin sensitivity (Zander *et al*., 2002) and GLP-1 extrapancreatic effects at the liver have been demonstrated (Burcelin *et al*., 2001), raising the possibility that some of the effects of liraglutide on insulin secretory function are mediated *via* extrapancreatic effects.

GLP-1 has been demonstrated to reduce food intake (Flint *et al*., 1998) by mechanisms involving both reduced gastric emptying (Willms *et al*., 1996) as well as effects on satiety and appetite (Flint *et al*., 1998). In accordance with its mechanism of action as a GLP-1 receptor agonist (Knudsen *et al*., 2000), liraglutide reduced food intake in the present study. Liraglutide also reduces food and water intake and body weight in normal rats (Larsen *et al*., 2001b). In the present study, the reduced food intake was initially accompanied by a reduction in body weight, relative to the vehicle-treated control animals. However, as the vehicle-treated rats became more severely diabetic, glucosuria and the resultant calorie loss meant that they stopped gaining any weight, in contrast to the less diabetic, liraglutide-treated animals. In this regard, it is worth noting that in diabetes-prevention studies with troglitazone and pioglitazone in ZDF rats, much greater weight gains compared to the liraglutide-treated animals in the present study have been observed (Sturis *et al*., 1995; Sreenan *et al*., 1996). This suggests that, despite the trend to gain weight caused by the improving diabetes and resultant reduction in calorie loss, liraglutide actually has a long-term weight-reducing effect in the ZDF rat. Similarly, studies with exendin-4 have demonstrated sustained reductions in weight (Greig *et al*., 1999; Szayna *et al*., 2000), while a study in man suggests that native GLP-1 given continuously over 6 weeks also causes weight loss (Zander *et al*., 2002). Reduced food intake *per se* has been demonstrated to affect diabetes development in the ZDF rat (Ohneda *et al*., 1995), leading us to carry out a second study utilizing pair feeding. We found that approximately half of the beneficial effect on glycemia associated with liraglutide treatment could be ascribed to a reduced food intake, confirming that the antihyperglycemic effects of liraglutide are likely to be mediated *via* more than one mechanism. These findings were corroborated by the results obtained in the 60% pancreatectomized rats in that liraglutide also reduced glucose AUC after an OGTT in that model, with pair-fed animals showing an intermediate response.

Several studies have demonstrated that agonists of the GLP-1 receptor, including native GLP-1, exendin-4, and liraglutide, are capable of enhancing β-cell mass, both *in vitro* and *in vivo*. The signal transduction mechanisms mediating these effects are incompletely understood, but involve upregulation of PDX-1 (MacDonald *et al*., 2002). Increased proliferation rate of existing β-cells, stimulation of neogenesis and inhibition of apoptosis have all been implicated in the mechanism by which the mass increases (Buteau *et al*., 1999; Edvell & Lindström, 1999; Xu *et al*., 1999; Perfetti *et al*., 2000; Bregenholt *et al*., 2001; Moldrup *et al*., 2001; Farilla *et al*., 2002; Rolin *et al*., 2002; Li *et al*., 2003). The majority of *in vivo* studies have thus demonstrated an upregulation of β-cell mass. In the present study, the difference in islet histology between ZDF rats treated for 2 and 6 weeks is striking, with an expected increased β-cell mass in animals treated for 6 weeks, but a surprising decrease in β-cell proliferation and mass when the treatment period was only 2 weeks. Whereas animals treated for 6 weeks were somewhat hyperglycemic, animals treated for only 2 weeks were still normoglycemic. Also, in normoglycemic, 60% pancreatectomized rats, we observed no effect on β-cell proliferation or mass after 4 days of treatment. It thus appears that the presence of normoglycemia may decrease the ability of liraglutide to increase β-cell mass. In support of our

findings, the mass of native β-cells was not found to differ between exendin-4 and control treatment in a study in normoglycemic athymic nude mice into which human fetal islets were transplanted, whereas increased mass and improved function of the β-cells in the graft of transplanted fetal islets were found (Movassat *et al*., 2002). However, other studies have indicated that exogenously administered GLP-1 (Perfetti *et al*., 2000) and exendin-4 (Xu *et al*., 1999) are able to increase β-cell mass in only slightly glucose intolerant (Perfetti *et al*., 2000) or in normoglycemic (Xu *et al*., 1999) rats. In those studies, drug delivery was discontinued several days prior to the assessment of β-cell mass, while our results were obtained during drug exposure. It is possible that a change in effect on β-cell mass occurs once compound exposure disappears, particularly since GLP-1 is known to stimulate somatostatin (d'Alessio *et al*., 1989), which in turn, has been shown to inhibit proliferation of RINm5F insulinoma cells (Stark & Mentlein, 2002). The differences between our results with liraglutide and those observed with GLP-1 and exendin-4 are unlikely to be due to a reduced ability of liraglutide to stimulate β-cell mass compared to GLP-1 and Exendin-4, since a recent study in *db*/*db* mice demonstrated liraglutide to be at least as efficacious as Exendin-4 on β-cell mass in that model (Rolin *et al*., 2002).

In conclusion, treatment with the long-acting GLP-1 derivative liraglutide attenuates diabetes development in prediabetic ZDF rats and has antihyperglycemic effects in 60% pancreatectomized rats. The effect is partly mediated by a reduction in food intake, but other features of liraglutide as a GLP-1 agonist are involved. In situations where normoglycemia is maintained, liraglutide does not cause expansion of β-cell mass, while on the longer term, liraglutide treatment confers protection of β-cell mass and function in ZDF rats, suggesting that the influence of GLP-1 agonism on β-cell mass dynamics *in vivo* may, directly or indirectly, depend on the glycemic state.

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