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Synergistic effect of the human GLP-1 analogue liraglutide and a dual PPARα/γ agonist on glycaemic control in Zucker diabetic fatty rats

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Aim/Hypothesis: Combination therapies are increasingly common in the clinical management of type 2 diabetes. We investigated to what extent combined treatment with the human glucagon-like peptide-1 (GLP-1) analogue liraglutide and the dual PPARα/γ agonist ragaglitazar would improve glycaemic control in overtly diabetic Zucker diabetic fatty (ZDF) rats.

Methods: Ninety overtly diabetic male ZDF rats were stratified into groups with matched haemoglobin A1c (HbA_{1c}) (9.0 ± 0.1%). Liraglutide (15 and 50 μg/kg subcutaneously twice daily), ragaglitazar (1 and 3 mg/kg perorally once daily) and their vehicles were studied as monotherapy and in combination in a 3 × 3 factorial design.

Results: After 4-week treatment, synergistic effects on HbA_{1c}, non-fasting morning blood glucose (BG) and/or 24-h BG profiles were observed with three of the four combinations. The relationship between plasma insulin and BG in combination-treated animals approached that of historical lean ZDF rats representing normal glucose homeostasis, suggesting that insulin secretion and insulin sensitivity were markedly improved. Increased insulin immunostaining in islets further supports the improved beta-cell function and/or insulin sensitivity in combination-treated animals. The synergistic effect on glycaemic control was found without a similar synergistic increase in beta-cell mass in the combination groups.

Conclusions/Interpretation: Our data demonstrate that combination treatment with a human GLP-1 analogue and a dual PPARα/γ agonist through distinct mechanism of actions synergistically improves glycaemic control in the ZDF rat.

Keywords: diabetes, liraglutide, Zucker diabetic fatty (ZDF) rat

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Introduction

Adequate glycaemic control of type 2 diabetic patients is difficult to achieve. Low and waning efficacy and/or risk of hypoglycaemia are limiting factors associated with current treatments. The typical type 2 diabetic patient is insulin resistant, exhibits abnormalities in glucose-stimulated insulin secretion and has deteriorating beta-cell function with time [1,2]. An ideal treatment would reverse all three of these defects. Both insulin secretagogues and insulin, including insulin analogues, possess inherent hypoglycaemic risks because these therapies do

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not completely restore the patients' normal feedback regulation that ensures tight glucose control present in non-diabetic individuals. By contrast, insulin sensitizers have a much lower intrinsic risk of hypoglycaemia because they facilitate the action of the prevailing insulin.

The use of combination therapy has become increasingly common in the clinical management of type 2 diabetes in order to compensate for the suboptimal efficacy of individual agents. However, in spite of efforts to design drugs that restore normal physiology, the currently available combinations that involve either an insulin or an insulin secretagogue all carry some risk of hypoglycaemia, which is a limiting factor for obtaining ideal glycaemic control. Glucagon-like peptide-1 (GLP-1) analogues and dual PPARα/γ agonists represent two novel classes of investigational compounds that possess low risks of hypoglycaemia. Furthermore, GLP-1 and GLP-1 analogues, including the GLP-1 analogues liraglutide and exenatide, have been shown to increase beta-cell mass in experimental animal models [3–5]. In clinical trials, GLP-1 receptor agonists have been shown to improve glycaemic control with a very low risk of hypoglycaemia [6–8]. Furthermore, this class of agents has demonstrated weight-reducing potential in clinical studies [6,7]. The dual PPARα/γ agonist ragaglitazar has also been shown to provide blood glucose (BG) and haemoglobin A1c (HbA_{1c}) lowering in type 2 diabetic patients [9], but known side-effects [e.g. water retention, body weight (BW) gain and unfavourable haematological changes] may limit the therapeutic potential of this investigational class. Notably, and as expected, episodes of hypoglycaemia were few.

In animal monotherapy studies, both GLP-1 receptor agonists [3] and PPARα/γ agonists [10] have significant antihyperglycaemic effects in male Zucker diabetic fatty (ZDF) rats, a commonly used model of type 2 diabetes with defects in both insulin secretion and action. However, normalization of glycaemic control was obtained with neither liraglutide [3] nor ragaglitazar when administered to overtly diabetic animals [10]. In the present study, we investigated the effect of combined treatment with liraglutide and ragaglitazar on glycaemic control in overtly diabetic ZDF rats and to what extent additive or perhaps even synergistic effects could be detected. Furthermore, in light of previously demonstrated effects of GLP-1 and PPARγ agonism on beta-cell mass, beta-cell function and beta-cell neogenesis [3–5,11–13], we performed histological analysis of the pancreata evaluating potential effects on beta-cell mass, beta-cell proliferation and insulin and pancreatic-duodenal homeobox-1 (PDX-1) immunoreactivity.

Methods

This animal study followed 'Principles of laboratory animal care' (National Institutes of Health publication number 85-23, revised 1985), and all animal experimental procedures were approved by the Danish Animal Experiments Inspectorate.

Animals

One hundred and eleven-week-old male ZDF rats (Genetic Models, Indianapolis, IN, USA) were housed (two rats per cage) and studied in the Animal Unit under ambient controlled conditions following a 12 : 12-h light : dark cycle (light on at 06:00 hours). Animals had free access to Purina 5008 chow (Purina Mills, Richmond, IN, USA) and fresh tap water.

Study Overview

The study was initiated in overtly diabetic male ZDF rats at 14 weeks of age. Prior to initiating the treatment, HbA_{1c}, non-fasting BG and plasma insulin (PI) concentrations were measured in 100 ZDF rats. The 90 rats with the highest HbA_{1c} values were selected for further study and allocated into nine treatment groups with matching HbA_{1c} levels. Treatment lasted for 30–31 days, during which time BG, PI, BW and communal (two rats per cage) food and water intake were measured. HbA_{1c} was measured again on the last treatment day. On that same day, the rats were injected with bromodeoxyuridine (BrdU) and killed 4 h later for removal of the pancreas for histological examination.

Treatment Groups

Treatment with two doses of liraglutide and two doses of ragaglitazar were studied as monotherapy and in combination in a 3×3 factorial design. Liraglutide was given in a lower dose (L_{lo}: 15 μg/kg) and a higher dose (L_{hi}: 50 μg/kg, which is the maximally tolerable dose in overtly diabetic ZDF rats without titration) or its vehicle (L_{veh}) subcutaneously twice daily (b.i.d.). Twice-daily dosing was necessary because of the shorter half-life of liraglutide in rats [3] compared with humans where it is once daily. Ragaglitazar was given in a lower (R_{lo}: 1 mg/kg) and a higher (R_{hi}: 3 mg/kg) dose or its vehicle (R_{veh}) perorally by gavage once daily. Doses were selected based on previous studies [3,10] and additional pilot studies and were adjusted to BW once weekly.

Assays

BG concentrations were measured by the immobilized glucose oxidase method using 10 μl whole blood diluted

in analysis solution (EBIO Plus autoanalyser and solution; Eppendorf, Hamburg, Germany). HbA_{1c} was measured using 5 µl whole blood diluted in analysis solution (COBAS MIRA Plus autoanalyser; Roche Diagnostic Systems, Basel, Switzerland). PI concentrations were measured with an in-house enzyme-linked immunosorbent assay method [14] using 15 µl specimens and GP114 and GP116 as primary and secondary antibodies, respectively, and rat insulin as standard.

Immunohistochemistry

The pancreata were fixed in 4% (v/v) paraformaldehyde, fractionated by the 'smooth fractionator method' [15] and embedded in paraffin. Sections were immunostained for insulin and BrdU or for non-beta cells (glucagon, somatostatin and pancreatic polypeptide) as previously described [3]. For PDX-1 and insulin double immunofluorescence staining, the following protocol was used: heat-induced antigen retrieval was performed in 0.01 M citrate buffer, pH 6.0. After blocking of non-specific binding, sections were incubated with rabbit anti-rat PDX-1 antibody (kindly provided by Dr O. D. Madsen, Hagedorn Research Institute, Gentofte, Denmark), biotinylated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Vectastain ABComplex (Vector, Burlingame, CA, USA), biotinylated tyramide (TSA indirect, NEL 700, NEN; Perkin Elmer Life Science, Boston, MA, USA) and Texas Red–conjugated streptavidin (Amersham Life Science, Buckinghamshire, UK). Subsequently, sections were incubated with guinea-pig anti-insulin antibody (MP Biomedicals, Irvine, Ca, USA) and Cy2-conjugated donkey anti–guinea pig immunoglobulin G (706-225-148; Jackson). An Olympus BX51 epifluorescence microscope equipped with selective Texas Red and fluorescein isothiocyanate filters and a DP50 digital camera (Olympus, Albertslund, Denmark) was used for analysis.

Statistical Evaluation of Data

The stereological estimation of beta-cell and non–beta cell mass and BrdU proliferation index was carried out as described earlier [3]. Data are presented as mean ± s.e.m. for n = 9–10 per group. As an exception, results on food and water intake represent n = 4–5 cages (two rats per cage). One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for groupwise comparisons was used for evaluation of the effects of the monotherapies. Two-way ANOVA was employed to test level of significance for the synergistic interaction of the two drugs above an additive effect. The theoretical additive effect for each drug combination was calculated as the sum of the effect of the individual monotherapies. For HbA_{1c}, the difference between post- and pretreatment levels (ΔHbA_{1c}) was used as summary measure for the statistical evaluation. A p value of <0.05 was considered statistically significant.

Results

Glycaemic Control

The development of non-fasting morning BG levels during the treatment period is shown in figure 1. The four combination treatments as well as monotherapy with $L_{veh} + R_{hi}$ markedly decreased BG levels. In general, treatment efficacy measured by this parameter was greatest after treatment for 1–2 weeks and thereafter efficacy declined. However, the $L_{hi} + R_{hi}$ combination group showed evidence of sustained efficacy on non-fasting morning BG levels. HbA_{1c} at the end of treatment (figure 2) was significantly reduced by the monotherapies with $L_{hi} + R_{veh}$, $L_{veh} + R_{lo}$ and $L_{veh} + R_{hi}$ ($p < 0.001$). Additionally, there was a significant interaction between combination treatments with $L_{lo} + R_{lo}$ and $L_{hi} + R_{lo}$ for HbA_{1c} (figure 2a, b), that is the effect of the combination was synergistic. For the other two combination treatments $L_{lo} + R_{hi}$ and $L_{hi} + R_{hi}$, at least additive effects were observed. Similar synergistic and

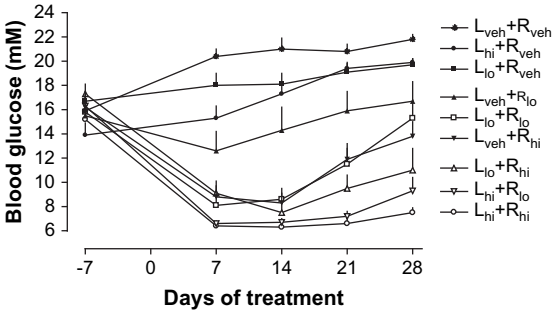

Fig. 1 BG during treatment. Non-fasting morning BG levels obtained 7 days prior to and once weekly during the treatment period. Data are presented as mean ± s.e.m. for n = 9–10 rats per group. BG, blood glucose;
$L_{veh} + R_{veh}$, liraglutide vehicle + ragaglitazar vehicle;
$L_{lo} + R_{veh}$, liraglutide lower dose + ragaglitazar vehicle;
$L_{hi} + R_{veh}$, liraglutide higher dose + ragaglitazar vehicle;
$L_{veh} + R_{lo}$, liraglutide vehicle + ragaglitazar lower dose;
$L_{veh} + R_{hi}$, liraglutide vehicle + ragaglitazar higher dose;
$L_{lo} + R_{lo}$, liraglutide lower dose + ragaglitazar lower dose;
$L_{lo} + R_{hi}$, liraglutide lower dose + ragaglitazar higher dose;
$L_{hi} + R_{lo}$, liraglutide higher dose + ragaglitazar lower dose;
$L_{hi} + R_{hi}$, liraglutide higher dose + ragaglitazar higher dose.

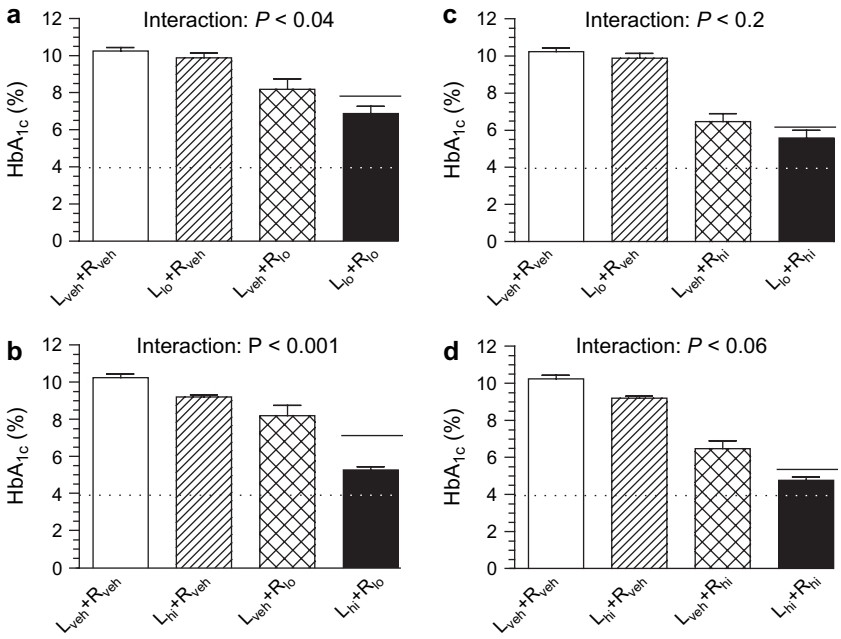

Fig. 2 HbA_{1c} at the end of treatment. Effects of liraglutide (hatched bars) and ragaglitazar (checked bars) monotherapies and combination therapies (black bars) compared with vehicle treatment (white bars) on post-treatment HbA_{1c} levels. Horizontal dotted lines, historical 12-week-old lean ZDF rats. Solid horizontal lines, theoretical additive effects of the monotherapies. Two-way ANOVA used for test for synergistic interaction of the two drugs above an additive effect. Abbreviations for treatment groups as in caption of figure 1. ANOVA, analysis of variance; HbA_{1c}, haemoglobin A1c; ZDF, Zucker diabetic fatty. Panels a–d are explained in the text.

additive effects of the combination therapies were observed on the integrated 24-h BG profiles obtained at the end of treatment (data not shown).

Food and Water Intake and BW

Table 1 shows food intake, water consumption and changes in BW measured after 22 days of treatment. As monotherapy, ragaglitazar treatment resulted in borderline significant increase in food intake; the higher dose of liraglutide tended to decrease food intake, and no interaction between compounds was observed. Water consumption showed treatment-related reductions at the high-dose level of both compounds with additive effects observed with combination therapy. In monotherapy, liraglutide dose-dependently reduced and ragaglitazar increased BW. The effects on BW in the $L_{hi} + R_{hi}$ and $L_{hi} + R_{lo}$ combination groups were significantly different from additive effect.

BG vs. PI Relationship

Figure 3 shows the relationship between PI and BG levels obtained from various groups of non-fasted rats and illustrates the age-dependent progressive deterioration of insulin secretion from the compensated prediabetic state observed in historical young rats to the more severely diabetic states seen during the vehicle treatment of the older ZDF rats in the present study. With an increasing degree of improvement obtained by the monotherapies and combination therapies at the end of the treatment period, the relationship between PI and BG approaches the relationship observed in historical 12-week-old lean ZDF rats representing normal glucose homeostasis for the age. This suggests that both insulin secretion and insulin sensitivity are markedly improved.

Insulin and PDX-1 Immunofluorescence Staining

Pancreatic sections were double immunofluorescence stained for insulin and PDX-1. As shown in merged pictures of the insulin (figure 4a, d, g and j) and PDX-1 (figure 4b, e, h and k) immunostaining, beta-cell nuclei in all groups expressed PDX-1 (figure 4c, f, i and l). Compared with the weak insulin staining observed in pancreatic islets from the vehicle group (figure 4a), insulin staining intensities were slightly increased in the $L_{hi} + R_{veh}$ and unaltered in the $L_{veh} + R_{hi}$ monotherapy

Table 1 Changes in BW and communal food and water intake measured at the end of the treatment period

Treatment groups	Communal food intake* (gram chow/2 rats per 24 h)		Communal water intake* (gram water/2 rats per 24 h)		Change in BW† (g)	
	Mean ± s.e.m. (four to five cages/gr)	Calculated additive effect	Mean ± s.e.m. (four to five cages/gr)	Calculated additive effect	Mean ± s.e.m. (9–10 rats/g)	Calculated additive effect
$L_{veh} + R_{veh}$	68 ± 5	—	269 ± 16	—	28 ± 2	—
$L_{lo} + R_{veh}$	69 ± 4	—	232 ± 19	—	19 ± 2	—
$L_{hi} + R_{veh}$	56 ± 3	—	179 ± 11‡	—	14 ± 2	—
$L_{veh} + R_{lo}$	77 ± 4	—	245 ± 27	—	95 ± 7§	—
$L_{veh} + R_{hi}$	83 ± 2	—	166 ± 14‡	—	141 ± 7§	—
$L_{lo} + R_{lo}$	72 ± 3	77	153 ± 16	208	107 ± 7	86
$L_{lo} + R_{hi}$	81 ± 2	83	134 ± 19	129	137 ± 6	133
$L_{hi} + R_{lo}$	69 ± 2	64	98 ± 5	155	103 ± 5¶	82
$L_{hi} + R_{hi}$	70 ± 2	70	94 ± 3	77	110 ± 4¶	128

ANOVA, analysis of variance; BW, body weight.
Abbreviations for treatment groups as in caption of figure 1.
*Twenty-four-hour communal food or water intake was measured after 3 weeks of treatment.
†The change in BW is calculated as post- minus pretreatment values.
‡$p < 0.05$ vs. vehicle (one-way ANOVA).
§$p < 0.005$ vs. vehicle (one-way ANOVA).
¶$p < 0.05$; interaction term (two-way ANOVA).

groups (figure 4d, g respectively). In contrast, the intensity of insulin staining in islets from the $L_{hi} + R_{hi}$ combination group (figure 4j) was markedly increased compared with both the vehicle and the monotherapy groups. Compared with the weak nuclear and cytoplasmic PDX-1 immunoreactivity observed in pancreatic beta cells from the vehicle group (figure 4b), the intensity of nuclear PDX-1 immunostaining was markedly upregulated by both $L_{hi} + R_{veh}$ and $L_{veh} + R_{hi}$ mono treatments and their $L_{hi} + R_{hi}$ combination (figure 4e, h and k).

Beta-cell and Non–beta Cell Mass

Stereological estimations of beta-cell and non–beta islet cell masses were performed. In the monotherapy groups treated with either dose of liraglutide or $L_{veh} + R_{lo}$, the beta-cell masses were similar to those of the vehicle group (figure 5a–c), whereas the $L_{veh} + R_{hi}$ monotherapy significantly ($p < 0.05$) decreased beta-cell mass (figure 5 d). No synergistic interaction between liraglutide and ragaglitazar was identified (figure 5a–d). There was no effect of the four different monotherapies on non–beta islet cell mass (data not shown). However, a significant ($p < 0.001$) interaction was observed in the $L_{hi} + R_{hi}$ combination group resulting in a non–beta islet cell mass higher than the expected additive effect of the two drugs (data not shown). The overall beta-cell proliferation index (as determined by immunostaining for BrdU incorporation into nuclei of insulin-positive islet cells was not affected by any of the treatments (data not shown).

Discussion

Our data demonstrate that combination therapy with the two compounds liraglutide and ragaglitazar exerting their actions on glucose homeostasis through distinct mechanisms results in highly efficacious BG and HbA_{1c} lowering after 4-week treatment of overtly diabetic male ZDF

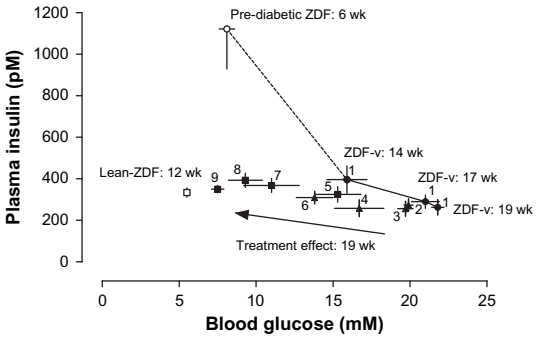

Fig. 3 Non-fasting BG–PI relationship. PI vs. BG levels for treatment groups included in this study (black symbols), for historical groups of 12-week-old lean ZDF rats (white square) and 6-week-old ZDF rats (white circle) for comparison to the normal and prediabetic, hyperinsulinemic conditions respectively. Listed and numbered one to nine, the groups indicated by black circles (vehicle treated ZDF at 14, 17 and 19 weeks of age), triangles (monotherapies) and squares (combination therapies) are as follows: 1, $L_{veh} + R_{veh}$; 2, $L_{hi} + R_{veh}$; 3, $L_{lo} + R_{veh}$; 4, $L_{veh} + R_{lo}$; 5, $L_{lo} + R_{lo}$; 6, $L_{veh} + R_{hi}$; 7, $L_{lo} + R_{hi}$; 8, $L_{hi} + R_{lo}$; 9, $L_{hi} + R_{hi}$. Abbreviations for treatment groups as in caption of figure 1. BG, blood glucose; PI, plasma insulin; ZDF, Zucker diabetic fatty.

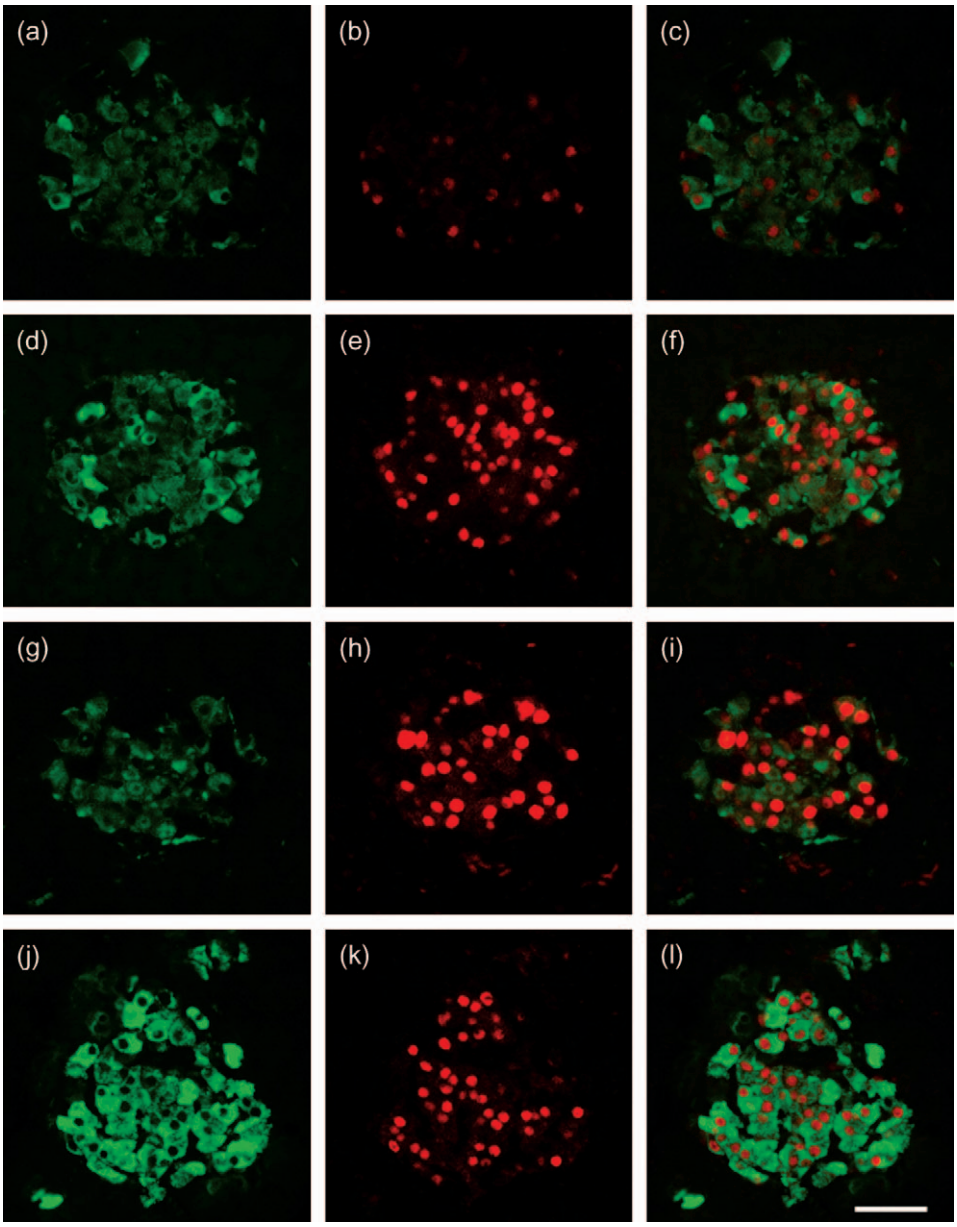

Fig. 4 PDX-1 and insulin immunostaining of mature islets. Sections double immunofluorescence stained for insulin (green) (a, d, g and j) and PDX-1 (red) (b, e, h and k). Merged images of insulin and PDX-1 stainings (c, f, i and l). (a–c) $L_{veh} + R_{veh}$; (d–f) $L_{hi} + R_{veh}$; (g–i) $L_{veh} + R_{hi}$; (j–l) $L_{hi} + R_{hi}$. Bar = 50 μm (l). Abbreviations for treatment groups as in caption of figure 1. PDX-1, pancreatic-duodenal homeobox-1.

rats. The effect was at least additive and for many effects directly synergistic, that is statistically significantly greater than the added effects of the two compounds in monotherapy. The synergistic effect on glycaemic control was most clear with the $L_{hi} + R_{lo}$ combination, where the observed reduction in HbA_{1c} was almost 5%, significantly more than the HbA_{1c} reduction of 3.1% that would have been expected if the effects had been only additive. Only borderline significant synergy was observed on HbA_{1c} with the combination of the higher doses of the two compounds, probably because of the large reduction in HbA_{1c} obtained with high-dose ragaglitazar monotherapy that minimizes the room for further improvement.

Liraglutide decreases food intake and attenuates BW gain in ZDF rats [3]. PPAR agonists such as rosiglitazone

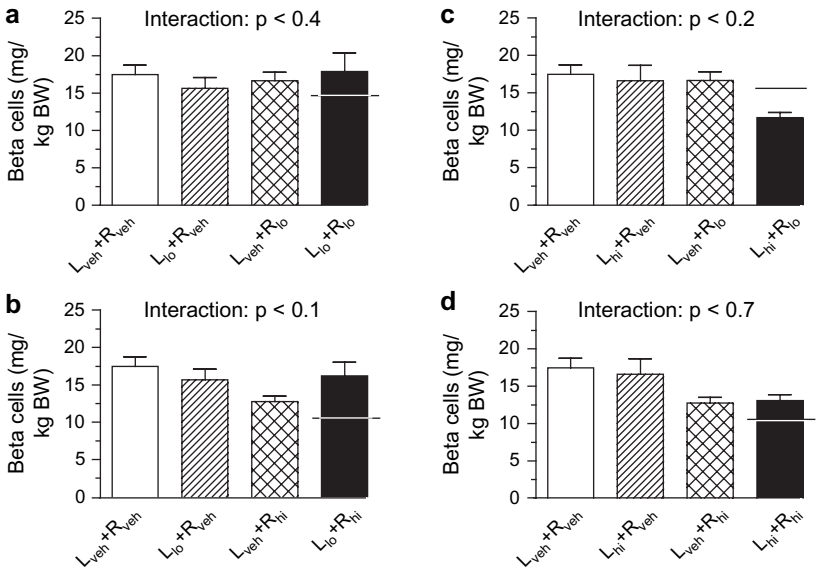

Fig. 5 Stereological evaluation of beta-cell mass. Effects of liraglutide (hatched bars) and ragaglitazar (checked bars) monotherapies and combination therapies (black bars) compared with vehicle treatment (white bars) on post-treatment beta-cell mass. Solid horizontal lines, theoretical additive effects of the monotherapies. Two-way ANOVA used for test for synergistic interaction of the two drugs above an additive effect. Abbreviations for treatment groups as in caption of figure 1. ANOVA, analysis of variance. Panels a–d are explained in the text.

and ragaglitazar are known to increase food intake in the ZDF rat [10,16], and ragaglitazar also borderline increased food intake in the present study. The effect on food intake of combining the two drugs was additive, making it unlikely that the observed synergies on efficacy were related to the observations in food. The data on BW are somewhat difficult to interpret. It is important to keep in mind that untreated or poorly controlled diabetes is a catabolic condition associated with a loss of calories because of glucosuria, thereby contributing independently to retarded body growth or even weight loss. Although we do not have any direct data on glucosuria in the present study, increased water intake is a good indicator. Pilot studies performed in-house indicate that 20–30% of the calories consumed over 24 h are lost through glucosuria in untreated, severely diabetic ZDF rats (unpublished observations). Thus, good metabolic control will in itself tend to increase BW because more consumed calories are retained. In spite of the far superior glycaemic control observed with the $L_{hi} + R_{hi}$ combination treatment, the effect of liraglutide in this group resulted in attenuated weight gain compared with the group given higher dose of ragaglitazar in monotherapy.

We did not assess beta-cell function or insulin sensitivity with tests such as glucose clamps or intravenous or oral glucose tolerance tests. However, the close proximity of the PI–BG relationship to normal, with near normalization of BG for the same levels of PI, makes it very likely that both beta-cell function and insulin sensitivity were greatly improved in the group treated with the $L_{hi} + R_{hi}$ combination. This is consistent with the ideas behind the classical homeostasis model assessment indices of insulin resistance and beta-cell function [17]. The increased insulin staining in the combination groups, most likely reflecting increased insulin content, is taken as a further indication of ameliorated beta-cell function, even though beta-cell mass was not markedly increased.

An increased insulin sensitivity caused by the ragaglitazar treatment of the ZDF rats [10] may indirectly have contributed to the improvement of beta-cell function because of relief from lipotoxicity and glucotoxicity as previously demonstrated by Unger and co-workers to be an important mechanism for beta-cell pathogenesis in this animal model [11,18]. In addition to the glucose-lowering actions, we know that both ragaglitazar [10] and liraglutide (unpublished data) treatments in monotherapy affect plasma lipid parameters in ZDF rats. The present lack of ragaglitazar-induced increase in insulin staining intensity is in contrast to our earlier studies with ragaglitazar and rosiglitazone in ZDF rats [10]. This is, however, most likely because of the fact that the ZDF rats used in the present study were older and overtly diabetic at the initiation of the therapy, whereas our

earlier results were obtained in animals that were either prediabetic or only moderately diabetic at the beginning of the treatment. In the latter study, ragaglitazar and rosiglitazone treatments normalized glycaemia, whereas the two monotherapies with ragaglitazar in the present study were unable to restore normoglycaemia.

GLP-1 has previously been demonstrated to induce PDX-1 expression in glucose-intolerant rats [19]. Prevention of progressive hyperglycaemia in ZDF rats with the PPARγ agonist troglitazone resulted in partial preservation of insulin and PDX-1 gene expression, whereas no effect was observed with troglitazone treatment of normoglycaemic lean ZDF control animals [20]. Moreover, PDX-1 expression was recently reported to be required for stimulation of insulin secretion by the GLP-1 receptor agonist exendin-4 [21]. These data suggest that GLP-1 and PPARγ agonists improve beta-cell function in part through effects on PDX-1. Supporting this hypothesis, we observed in the present study a marked increase in PDX-1 immunostaining intensity in mature islets from animals treated with liraglutide and ragaglitazar either alone or in combination.

Recently, similar effects have been reported in ZDF rats using a combination of liraglutide and the PPARγ agonist pioglitazone [22]. Compared with those data, the present results were obtained with much lower doses of liraglutide. Thus, whereas the synergistic interactions in the paper by Larsen *et al*. [22] were obtained using 200 μg/kg b.i.d., we observed synergy at doses of 15 and 50 μg/kg b.i.d. This shows that synergy between liraglutide and compounds with PPARγ agonist activity can be expected across a broad range of liraglutide doses. We do, however, not know whether the additional PPARα activity of ragaglitazar plays a role in the doses of liraglutide at which synergy can be observed. It is noteworthy that either tripling the dose of ragaglitazar in monotherapy (from lower to higher) or quadrupling the dose of liraglutide in monotherapy (comparing the higher dose in the current study with that utilized in the paper by Larsen *et al*.) resulted in less improvement in HbA_{1c} than combining those doses of the two compounds as demonstrated by the $L_{hi} + R_{lo}$ combination in the present study, further highlighting the strength of the synergy of the liraglutide/ragaglitazar combination.

In summary, we have demonstrated synergistic effects of liraglutide and ragaglitazar treatments in overtly diabetic ZDF rats on glycaemic control. The identification of such synergistic interactions between antidiabetic agents used in the treatment of type 2 diabetic patients could have important implications for the potential to alter disease progression, particularly if the mechanism involves long-term improvement of beta-cell function.

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