

Pharmacological reduction of NEFA restores the efficacy of incretin-based therapies through GLP-1 receptor signalling in the beta cell in mouse models of diabetes

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Abstract

Aims/hypothesis Type 2 diabetes mellitus is associated with reduced incretin effects. Although previous studies have shown that hyperglycaemia contributes to impaired incretin responses in beta cells, it is largely unknown how hyperlipidaemia, another feature of type 2 diabetes, contributes to impaired glucagon-like peptide 1 (GLP-1) response. Here, we investigated the effects of NEFA on incretin receptor signalling and examined the glucose-lowering efficacy of

incretin-based drugs in combination with the lipid-lowering agent bezafibrate.

Methods We used *db/db* mice to examine the in vivo efficacy of the treatment. Beta cell lines and mouse islets were used to examine GLP-1 and glucose-dependent insulinotropic peptide receptor signalling.

Results Palmitate treatment decreased *Glp1r* expression in rodent insulinoma cell lines and isolated islets. This was associated with impairment of the following: GLP-1-stimulated cAMP production, phosphorylation of cAMP-responsive elements binding protein (CREB) and insulin secretion. In insulinoma cell lines, the expression of exogenous *Glp1r* restored cAMP production and the phosphorylation of CREB. Treatment with bezafibrate in combination with des-fluoro-sitagliptin or exendin-4 led to more robust glycaemic control, associated with improved islet morphology and beta cell mass in *db/db* mice.

Conclusions/interpretation Elevated NEFA contributes to impaired responsiveness to GLP-1, partially through down-regulation of GLP-1 receptor signalling. Improvements in lipid control in mouse models of obesity and diabetes increase the efficacy of incretin-based therapy.

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Keywords Dipeptidyl peptidase-4 · Exendin-4 · Glucagon-like peptide 1 · Glucose-dependent insulinotropic polypeptide · Islet · NEFA · Non-esterified fatty acid · Receptor

Abbreviations

Ad	Adenoviral vector
Ad-GFP	Adenoviral vector with green fluorescent protein (GFP)
CREB	cAMP-responsive elements binding protein
D-GIP	[D-Ala ²]GIP(1-30)
DPP-4	Dipeptidyl peptidase-4
GFP	Green fluorescent protein

GIP	Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLP-1	Glucagon-like peptide 1
GLP1R	GLP-1 receptor
p-CREB	CREB phosphorylation
PPAR	Peroxisome proliferator-activated receptor
SREBP1c	Sterol regulatory element binding protein 1c

Introduction

The gastrointestinal hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), cause glucose-dependent insulin secretion from pancreatic beta cells within minutes of nutrient ingestion [1, 2]. One characteristic of type 2 diabetes mellitus is impaired incretin effect [3, 4], although the secretion of GIP and GLP-1 is not always decreased [4–6]. This indicates that the reduced incretin effect is due to defects in incretin receptor signalling pathways, rather than to the concentration of incretin hormones. The insulinotropic activity of GIP is largely impaired in patients with type 2 diabetes [4, 7]. In contrast, the insulinotropic effects of GLP-1 are partially preserved, which is important for its therapeutic potential, but insulin responses are substantially reduced, especially when studies are done at comparable glucose levels [7, 8]. Moreover, a growing body of evidence has shown that the glucose-lowering effects of GLP-1 are mediated by various mechanisms, including stimulation of glucose-dependent insulin secretion in pancreatic beta cells [1, 2], promotion of pancreatic beta cell proliferation and inhibition of beta cell apoptosis [9–11], inhibition of pancreatic alpha cell glucagon release [12, 13] and regulation of appetite and the central nervous system [2, 14]. These attributes of GLP-1 provide a strong basis for novel pharmacotherapies in type 2 diabetes. Currently, synthetic versions of GLP-1 mimetics (e.g. exenatide and liraglutide) and dipeptidyl peptidase-4 (DPP-4) inhibitors (e.g. sitagliptin and vildagliptin), which reduce GLP-1 and GIP degradation by DPP-4, have been approved for the treatment of type 2 diabetes [2, 15].

Type 2 diabetes develops as a result of impaired beta cell function and is closely associated with increased plasma NEFA, which are thought to be an important link between obesity and type 2 diabetes [16–18]. NEFA can result in a state of insulin resistance [19], induce pancreatic beta cell dysfunction and cause beta cell death [18, 20]. Although acute exposure to elevated plasma NEFA enhances glucose- and non-glucose-stimulated insulin secretion in vitro and in vivo [18, 21], long-term exposure to NEFA impairs glucose-stimulated insulin secretion [22]. Recently, it was reported that while incretin secretion is similar between obese and non-obese type 2 diabetic patients [23], obesity impairs the incretin effect

independently of glucose tolerance [24]. It has also been reported that loss of the incretin effects was more extensive in obese than in lean type 2 diabetic patients [25]. More recently, Bando et al reported that obesity may attenuate the HbA_{1c}-lowering effect of sitagliptin in Japanese type 2 diabetic patients [26]. This suggests that lipids may be involved in the regulation of incretin responsiveness in pancreatic beta cells. However, little is known about the influence of NEFA on incretin receptor signalling. Our previous study showed that hyperglycaemia downregulates GLP-1 receptor (GLP1R), which potentially contributes to the impaired incretin response in beta cells [27]. Furthermore, the normalisation of blood glucose concentrations improves the insulin response to GLP-1 and GIP in patients with type 2 diabetes [28]. In the present study, therefore, we used in vitro and in vivo approaches to investigate the role of NEFA in the impairment of incretin responses.

Methods

Chemicals and reagents For details, see electronic supplementary material (ESM), Chemicals and reagents.

Cell culture and treatment INS-1E cell line (passages 65 to 75) was a kind gift from P. Maechler (Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland). INS-1E and MIN6 cells were grown as described previously [11, 29]. Cells were cultured in 6- or 12-well plates for 24 to 48 h before treatment with palmitate or 1.05% (wt/vol) BSA (in RPMI 1640 or DMEM with 1% FBS). GLP-1, GIP, exendin-4 and [D-Ala²]GIP(1–30) (D-GIP) were dissolved in PBS and stored at –80°C. To achieve expression of exogenous *Glp1r*, cells were infected with an adenoviral vector (Ad), Ad-GLP1R, for 6 h prior to treatment with palmitate or 1.05% BSA.

Islet isolation and culture, construction of Ad-GLP1R, RT-PCR, western blotting and measurement of cAMP production, and insulin secretion Pancreatic islets were isolated from 8- to 9-week-old male C57BL/6J mice as previously described (ESM Methods, Isolation and cell culture). The gateway-compatible adenoviral expression system was used to generate the recombinant adenoviruses (ESM Methods, Construction of Ad-GLP1R). Real-time PCR (ESM Methods, RNA extraction and quantitative RT-PCR) and western blotting (ESM Methods, Analysis of phosphorylation of CREB) were performed with standard procedures. Intracellular cAMP content was determined using a kit (Cyclic AMP EIA; Cayman, Ann Arbor, MI, USA) (ESM Methods, Measurement of cAMP production) and protein levels were assayed by BCA for the correction. Insulin secretion was measured in INS1-E cells or mouse islets after exposure to 3 or 16.7 mmol/l glucose (ESM Methods, Measurement of insulin secretion).

Animals and experimental protocols All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by The Animal Subjects Committee of The Chinese University of Hong Kong. Male *db/db* and *db/+* (control) mice (aged 7 to 8 weeks) were obtained from The Chinese University of Hong Kong and housed in specific pathogen-free conditions with a 12 h light–dark cycle and free access to water and food. Experiments were performed after 1 week of acclimatisation. For drug treatments, des-fluoro-sitagliptin (200 mg/kg) and bezafibrate (100 mg/kg) were dissolved in 0.5% (wt/vol) CMC and given by gavage; exendin-4 (10 nmol/kg) and D-GIP (24 nmol/kg) were dissolved in PBS and given by intraperitoneal injection. Mice were treated daily (16:00 to 18:00 hours) by gavage or intraperitoneal injection for the indicated time. Fed random blood glucose was monitored weekly at 09:00 to 10:00 hours. For measurement of the acute glucose-lowering actions of exendin-4 and D-GIP, *db/db* mice were treated with vehicle or bezafibrate for 2 weeks and then injected intraperitoneally with saline, exendin-4 or D-GIP. Glucose levels were determined at 0, 30, 60 and 240 min after injection.

OGTT, insulin tolerance test and serum lipid profile measurement For the OGTT, mice were fasted overnight (~17 h). Glucose levels were determined using a glucometer (Johnson & Johnson, Milpitas, CA, USA) at 0, 30, 60 and 120 min after oral administration of 0.3 g/kg glucose. For the insulin tolerance test (ITT), done after 6 h of fasting, mice were intraperitoneally injected with 2 IU/kg human insulin (Novo Nordisk, Bagsvaerd, Denmark). Glucose levels were measured at 0, 30, 60 and 120 min after the injection. Triacylglycerol, NEFA and total cholesterol concentrations were measured using related kits (Wako Lab Assays, Richmond, VA, USA). HDL-cholesterol was determined by enzymatic assays using an automated analyser (Olympus, Tokyo, Japan).

Histological analysis Pancreases were quickly dissected from mice and fixed in 4% (wt/vol.) paraformaldehyde, after which paraffin-embedded 4- μ m sections were immunostained overnight at 4°C with guinea pig anti-insulin (Dako, Glostrup, Denmark) and mouse anti-glucagon (1:200; Accurate Chemical & Scientific, Westbury, NY, USA), or with mouse anti-BrdU (BD Biosciences, Franklin Lakes, NJ, USA) antibodies. Following this, staining with cy2-goat anti-guinea pig or cy3-donkey anti-mouse (1:400; Jackson, West Grove, PA, USA) was done at room temperature for 2 h. The sample slides were washed three times with 0.1% PBS Tween (vol./vol., PBST) and stained with DAPI (Invitrogen, Grand Island, NY, USA) before microscopic analysis. The insulin-positive area vs total pancreas or total islet area was evaluated using Image J (NIH, Bethesda, Maryland, USA) [10].

Statistical analysis Animal data are expressed as means \pm SEM. Differences between the groups were examined for statistical significance using one-way or two-way ANOVA, followed by Dunnett's post tests or *t* tests (as appropriate). For in vitro experiments, quantitative RT-PCR data are expressed as means \pm SEM; other data are presented as means \pm SD. Statistical significance was determined by Student's *t* test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Reduced expression of *Glp1r* and reduction of GLP-1-stimulated insulin secretion in palmitate-treated beta cells and mouse islets In rat INS-1E cells, palmitate treatment significantly reduced *Glp1r* mRNA expression and levels of GLP1R in a dose-dependent manner (Fig. 1a, ESM Fig. 1a, c), while no effect on *Gipr* mRNA expression was observed (Fig. 1a). Similar results were found in mouse MIN6 cells (Fig. 1b, ESM Fig. 1b, d). Consistent with these results, exposure to palmitate also led to decreases in *Glp1r* mRNA expression in isolated islets (Fig. 1c). We next examined transcription factor 7-like 2 (TCF7L2), which has been reported to regulate the expression of *Glp1r* and *Gipr* in beta cells [30]. However, *Tcf7l2* mRNA expression was not changed by palmitate treatment in INS-1E or MIN6 cells (Fig. 1a, b). Similarly, *Irs2* mRNA was not affected by palmitate (Fig. 1a, b). Upregulation of uncoupling protein-2 (UCP2) and sterol regulatory element binding protein 1c (SREBP1c) is known to play a crucial role in NEFA-induced beta cell dysfunction [31]. As expected, the mRNA expression of *Ucp2* and *Srebp1c* (also known as *Srebf1*) was significantly increased by palmitate in INS-1E cells (Fig. 1a). However, in MIN6 cells, *Ucp2* was upregulated while *Srebp1c* was unchanged by palmitate (Fig. 1b).

In islets isolated from *db/db* mice, we also found that *Glp1r* and *Gipr* mRNA were significantly reduced compared with expression in control mice (Fig. 1d, e). Interestingly, *db/db* mice that were treated with the lipid-lowering agent bezafibrate for 2 weeks displayed partial restoration of *Glp1r* and *Gipr* mRNA to levels not different from control mice (Fig. 1d, e), with lowered triacylglycerol and NEFA, but no obvious changes in glucose levels [32].

To further assess the functional consequences of the reductions in *Glp1r* and *Gipr* expression, we assayed insulin secretion in INS-1E cells and isolated islets. In INS-1E control cells, both GLP1R agonists (GLP-1, exendin-4) and the GIP receptor (GIPR) agonists (GIP, D-GIP) markedly increased insulin secretion in the presence of 16.7 mmol/l glucose (Fig. 1f). However, after pre-incubation with palmitate, this response was significantly attenuated (Fig. 1f). Similar results were found in isolated mouse islets, with palmitate

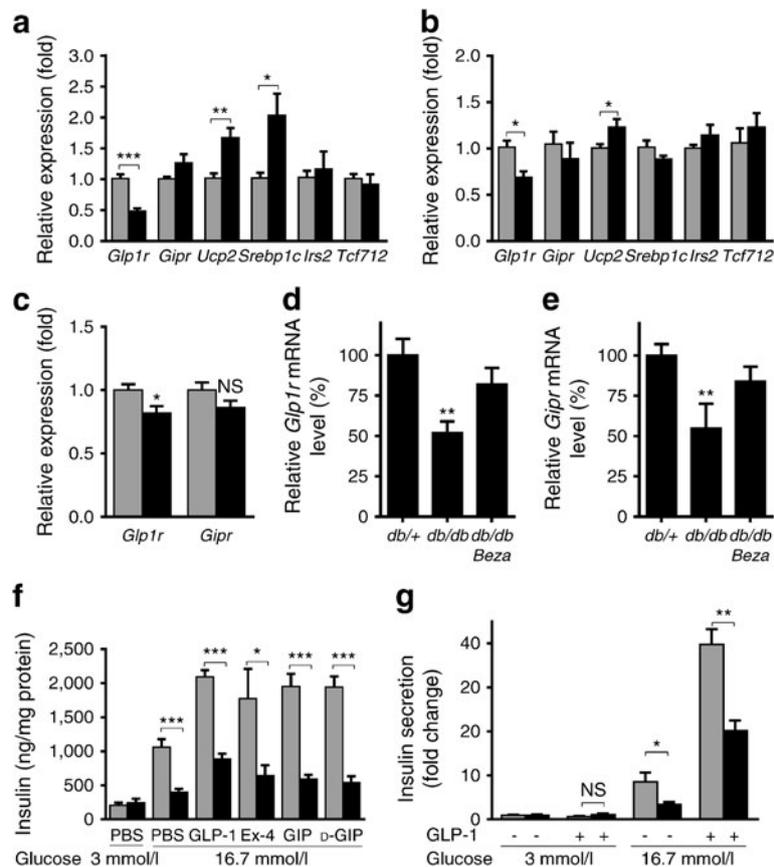


Fig. 1 *Glp1r* mRNA expression and GLP-1-stimulated insulin secretion are reduced in palmitate-treated rodent insulinoma cell lines and mouse islets. **(a)** INS-1E or **(b)** MIN6 cells were treated for 24 h with 1.05% BSA (grey bars) or palmitate (INS-1E 0.8 mmol/l, MIN6 0.4 mmol/l) (black bars), and the relative mRNA expression of genes as indicated was quantified by quantitative RT-PCR. β -Actin mRNA was used as control. Data were normalised to those for cells treated with BSA; $n=4-6$; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for palmitate- vs BSA-treated. **(c)** Mouse islets were treated for 48 h with 1.05% BSA or 0.4 mmol/l palmitate, and the relative mRNA expression of *Glp1r* and *Gipr* was quantified by quantitative RT-PCR. Data were normalised to those for islets treated with BSA; $n=5$; * $p<0.05$ vs BSA-treated

islets. **(d, e)** *db/db* mice were treated for 2 weeks with bezafibrate (Beza) (0.2% of food) and the relative mRNA expression of **(d)** *Glp1r* and **(e)** *Gipr* in islets from control (*db/+*), untreated *db/db* and bezafibrate-treated *db/db* mice was quantified; $n=3-8$ for each experimental group; ** $p<0.01$ vs islets from control. **(f)** INS-1E cells and **(g)** isolated islets were treated with 1.05% BSA or palmitate (INS-1E 0.8 mmol/l, islets 0.4 mmol/l) for 24 and 48 h, respectively. Glucose-stimulated insulin secretion was measured **(f)** in the presence of PBS, GLP-1 (100 nmol/l), exendin-4 (Ex-4; 100 nmol/l), GIP (100 nmol/l) or D-GIP (100 nmol/l) as described, and **(g)** as fold changes relative to the corresponding low-glucose (3 mmol/l) controls; $n=3-5$; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs corresponding BSA-treated controls

treatment decreasing the fold induction of GLP-1-stimulated glucose-dependent insulin secretion (Fig. 1g).

Palmitate impairs GLP-1-stimulated cAMP production and phosphorylation of cAMP-responsive elements binding protein in rodent insulinoma cell lines GLP-1 and GIP exert their effects by binding to their respective receptors, GLP1R and GIPR, leading to activation of adenylate cyclase and elevation of intracellular cAMP. In INS-1E cells, palmitate treatment decreased GLP-1- and GIP-stimulated cAMP production (Fig. 2a), while in MIN6 cells, GLP-1- but not GIP-stimulated cAMP production was reduced by palmitate (Fig. 2b). We then examined cAMP-responsive elements binding protein (CREB) phosphorylation (p-CREB), as CREB is an important transcription factor in glucose

homeostasis and beta cell survival [33]. In INS-1E cells, palmitate treatment significantly reduced GLP-1-stimulated p-CREB in a time- and dose-dependent manner (Fig. 2c, e). In contrast, GIP-stimulated p-CREB was unchanged throughout the time course of activation (Fig. 2c, f). Similarly, in MIN6 cells, palmitate treatment decreased GLP-1- but not GIP-stimulated p-CREB (Fig. 2d).

Expression of exogenous Glp1r restores GLP-1-stimulated cAMP production and p-CREB in palmitate-treated rodent insulinoma cell lines As GLP1R signalling is crucial for glucose homeostasis, we examined whether the expression of exogenous *Glp1r* could reverse the impairment of GLP1R signalling by palmitate. We constructed and expressed adenoviral vectors expressing mouse *Glp1r* (Ad-

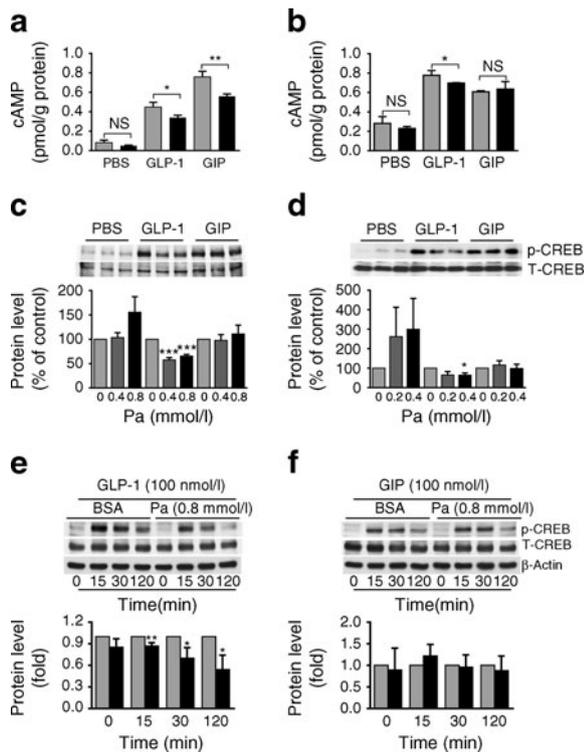


Fig. 2 Palmitate impairs GLP-1-stimulated cAMP production and phosphorylation of CREB in rodent insulinoma cell lines. (**a, c, e, f**) INS-1E or (**b, d**) MIN6 cells were treated with 1.05% BSA (grey bars [**a, b, c, d, e, f**] or palmitate (Pa); black bars 0.8 mmol/l [**a, c, e, f**], 0.4 mmol/l [**b, d**]; dark grey bars 0.4 mmol/l [**c**], 0.2 mmol/l [**d**] for 24 h) and stimulated with PBS, 100 nmol/l GLP-1 or 100 nmol/l GIP, after which (**a, b**) total cAMP, and (**c, d**) the dose- and (**e, f**) time-dependent phosphorylation of CREB (p-CREB) were assessed. Total cAMP (**a, b**) was measured as described (ESM Methods, Measurement of cAMP production) after cells had been stimulated for 10 min in medium containing 500 μ mol/l IBMX, in the presence of PBS, 100 nmol/l GLP-1 or 100 nmol/l GIP. (**c, d**) p-CREB was analysed by western blot after cells had been stimulated with PBS, 100 nmol/l GLP-1 or 100 nmol/l GIP for 15 min or (**e, f**) for the indicated times. Intensities were quantified, normalised against the level of actin and expressed as per cent of protein abundance in corresponding BSA-treated cells. Results shown are representative of three independent experiments; * p <0.05, ** p <0.01 and *** p <0.001 vs corresponding BSA-treated cells

GLP1R) in INS-1E and MIN6 cells. Our RT-PCR results show that mouse *Glp1r* was expressed in rat INS-1E cells after infection with Ad-GLP1R (ESM Fig. 2). Functionally, INS-1E cells infected with Ad-GLP1R also showed increases in basal levels of GLP-1-stimulated cAMP production compared with cells infected with a control Ad with green fluorescent protein (GFP) (Ad-GFP) (Fig. 3a). This result indicated enhanced GLP1R signalling. Palmitate treatment resulted in reduced GLP-1-stimulated cAMP production in INS-1E cells infected with Ad-GFP or Ad-GLP1R (Fig. 3a). However, Ad-GLP1R-mediated expression of exogenous *Glp1r* still restored GLP-1-stimulated cAMP production to the normal level in the presence of

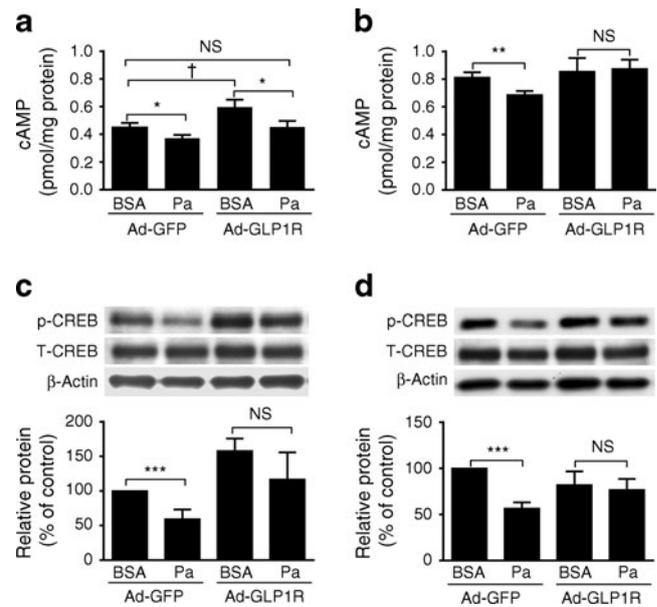


Fig. 3 Expression of exogenous *Glp1r* reversed the palmitate-induced impairment of GLP-1-stimulated cAMP production and p-CREB in rodent insulinoma cell lines. (**a, c**) INS-1E or (**b, d**) MIN6 cells were infected overnight with Ad-GFP and Ad-GLP1R, and then treated for 24 h with 1.05% BSA or 0.8 mmol/l palmitate (Pa). Total cAMP (**a, b**) and p-CREB (**c, d**) were assessed as for Fig. 2. The intensity of western blots was quantified, normalised against the level of actin and expressed as percentages of protein abundance in the corresponding BSA-treated and GFP-infected cells. Results are representative of three independent experiments; * p <0.05, ** p <0.01 and *** p <0.001 for palmitate vs corresponding BSA-treated cells; † p <0.05 for Ad-GLP1R vs GFP-infected cells treated with BSA

palmitate (Fig. 3a). Expression of exogenous *Glp1r* also restored GLP-1-stimulated p-CREB to the normal level in the presence of palmitate (Fig. 3c). Similarly, in MIN6 cells, infection with Ad-GLP1R protected against the palmitate-mediated reductions of GLP-1-stimulated cAMP production and p-CREB (Fig. 3b, d). To summarise, the above data suggest that the impairment of GLP-1-stimulated cAMP production and p-CREB in rodent insulinoma cell lines by palmitate can be at least partially restored by the expression of exogenous *Glp1r*.

Lipid lowering enhances the efficacy of the DPP-4 inhibitor, des-fluoro-sitagliptin, in db/db mice Since our findings implicate increased fatty acids in the impairment of GLP1R signalling through downregulation of GLP1R, we next investigated whether an improvement in the lipid profile would enhance responsiveness to incretin hormones in mouse models of diabetes. We first used the DPP-4 inhibitor, des-fluoro-sitagliptin, combined with the lipid-lowering drug, bezafibrate, in *db/db* mice. Administration of des-fluoro-sitagliptin or bezafibrate alone had no effect on blood glucose levels during the 8 week treatment period compared with the vehicle-treated control group (Fig. 4a, b). In

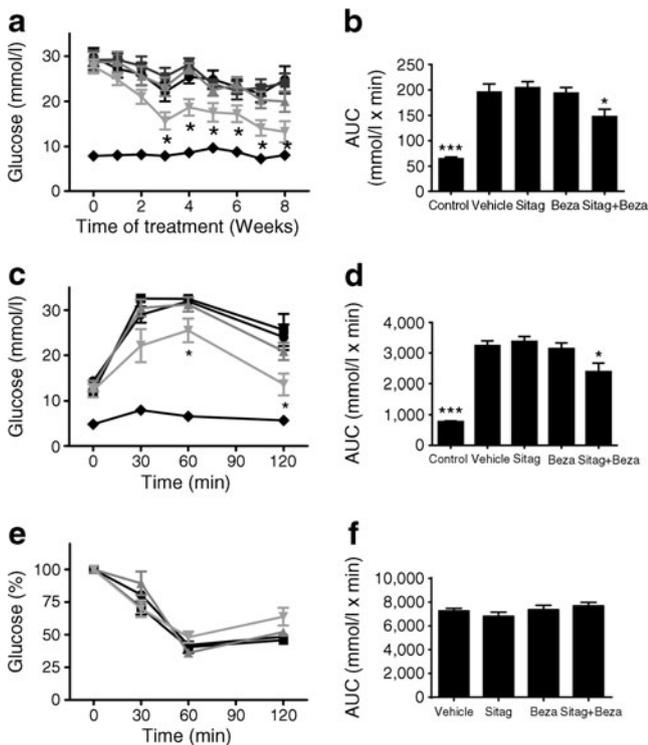


Fig. 4 Lipid lowering enhances the efficacy of the DPP-4 inhibitor des-fluoro-sitagliptin to improve glucose tolerance in *db/db* mice. Mice (*db/db*) were orally treated with des-fluoro-sitagliptin or bezafibrate alone, or with sitagliptin and bezafibrate combined. Treatment was once a day for 8 weeks. (a, b) Random fed blood glucose levels, and results of (c, d) an OGTT (after 6 weeks of treatment) and (e, f) an ITT (after 7 weeks of treatment) are shown. $n=5-8$ for each experimental group; $*p<0.05$ and $***p<0.01$ compared with vehicle group. Key: (a, c) black diamonds, control; black circles, vehicle; black squares, des-fluoro-sitagliptin; upright triangles (grey), bezafibrate; reversed triangles (grey), des-fluoro-sitagliptin plus bezafibrate; (e) black circles, vehicle; black squares, des-fluoro-sitagliptin; upright triangles (grey), bezafibrate; reversed triangles (grey), des-fluoro-sitagliptin plus bezafibrate

contrast, treatment with des-fluoro-sitagliptin and bezafibrate together significantly decreased blood glucose levels from 3 weeks onwards until the end of the experiment (Fig. 4a, b). We next examined the effects of these treatments on glucose tolerance by performing OGTTs. Glucose tolerance in *db/db* mice was not affected by 6 weeks of treatment with des-fluoro-sitagliptin or bezafibrate alone (Fig. 4c, d). However, treatment with des-fluoro-sitagliptin and bezafibrate combined significantly improved glucose excursions during the OGTT compared with the vehicle control group (Fig. 4c, d). This was not due to changes in insulin sensitivity, as there was no difference in the blood glucose response between the treatment groups during the ITT (Fig. 4e, f). The lipid-lowering effects of bezafibrate were confirmed by measuring circulating triacylglycerol and NEFA concentrations. As expected, serum triacylglycerol and NEFA were significantly decreased after bezafibrate and combined des-fluoro-sitagliptin and bezafibrate treatments (ESM

Fig. 3). In addition, HDL and total cholesterol levels were elevated in the bezafibrate and combined des-fluoro-sitagliptin and bezafibrate groups (ESM Fig. 3).

Pancreatic beta cells are important targets of incretin-based drugs. We analysed the islet morphology and beta cell mass of *db/db* mice after the various drug treatments. As shown in Fig. 5a, the normal islet architecture of control mice comprised a large core of insulin-positive beta cells ringed by a mantle of glucagon-positive alpha cells. In contrast, islets of vehicle-treated *db/db* mice exhibited an abnormal architecture, with reduced beta cells and increased alpha cells that were scattered throughout the islet core (Fig. 5a). A similar abnormal islet morphology was observed in *db/db* mice that were treated with des-fluoro-sitagliptin or bezafibrate alone (Fig. 5a). Only mice treated with a combination of des-fluoro-sitagliptin and bezafibrate displayed an improved islet architecture approaching that of normal islets, as evidenced by increased beta cell mass (measured as beta cell area per pancreas area) (Fig. 5b), an increased proportion of beta cells per islet (Fig. 5c) and the appearance of normal beta cell distribution within the islets (Fig. 5a).

Lipid lowering enhances the efficacy of an agonist to GLP1R (exendin-4) but not to GIPR (D-GIP) in db/db mice DPP-4 inhibition prevents the degradation of GLP-1 and GIP, resulting in increased levels of biologically active GLP-1 and GIP in vivo. To investigate the effects of lipid lowering on the activity of each incretin separately, we tested the efficacy of the GLP1R agonist, exendin-4, or the

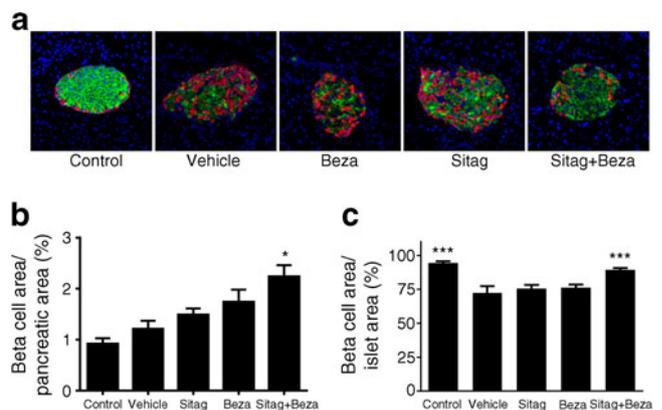


Fig. 5 Immunohistochemical analysis of the pancreas of *db/db* mice after co-treatment with the DPP-4 inhibitor des-fluoro-sitagliptin and bezafibrate. Mice (*db/db*) were orally treated with des-fluoro-sitagliptin (Sitag) or bezafibrate (Beza) alone, or with sitagliptin and bezafibrate combined (Sita + Beza). Treatment was once a day for 8 weeks. (a) Representative images of immunofluorescence analysis of islets stained for insulin (green), glucagon (red) and DAPI (blue). (b) Beta cell mass expressed as the percentage of insulin-positive area to total pancreas area and (c) ratios of insulin-positive beta cell area to total islet area were assessed from the staining for insulin and glucagon in images (a). $n=7-12$ for each experimental group; $*p<0.05$ and $***p<0.001$ compared with vehicle groups

GIPR agonist, D-GIP, either alone or in combination with bezafibrate, in *db/db* mice. First, *db/db* mice were treated with vehicle or bezafibrate for 2 weeks. As expected, these treatments significantly reduced plasma triacylglycerol and NEFA levels (data not show) without changes in blood glucose levels (Fig. 6c, d). We then assessed the glucose-lowering effect of acute GLP1R or GIPR activation after an intraperitoneal injection of a single dose of exendin-4 (1 nmol/kg) or D-GIP (24 nmol/kg). Acute treatment of *db/db* mice with exendin-4 significantly lowered blood glucose to a similar level to that in vehicle- and bezafibrate-treated groups compared with PBS injection (Fig. 6a). In contrast, the administration of D-GIP did not affect the hyperglycaemic status in either vehicle- or bezafibrate-treated *db/db* mice (Fig. 6b). Following the acute glucose-lowering experiments, the mice were treated with exendin-4 or D-GIP for an additional 3 weeks as described in the Methods. Under these chronic experimental conditions, neither exendin-4 nor D-GIP administration alone altered blood glucose levels (Fig. 6c, d). In mice treated with exendin-4 and bezafibrate

combined, fed blood glucose levels were significantly reduced after 1 week and for the remainder of the treatment period (Fig. 6c). In contrast, treatment with D-GIP and bezafibrate combined did not affect blood glucose levels (Fig. 6d). In response to the OGTT only the group treated with exendin-4 and bezafibrate combined exhibited significantly improved glucose tolerance (Fig. 6e). In contrast, D-GIP slightly worsened glucose excursions during the OGTT and combined treatment with D-GIP and bezafibrate did not improve glucose tolerance (Fig. 6f). Chronic treatment of *db/db* mice with bezafibrate, exendin-4 or exendin-4 and bezafibrate combined did not alter insulin sensitivity, as indicated by the unchanged blood glucose levels compared with the vehicle control group during the ITT (ESM Fig. 4a). On the other hand, in mice treated with D-GIP or D-GIP and bezafibrate combined, the AUC for blood glucose levels during the ITT was slightly, but significantly elevated, indicating a modest impairment of insulin action (ESM Fig. 4b). As expected, exendin-4 or D-GIP treatments had no effects on serum triacylglycerol, NEFA and HDL-

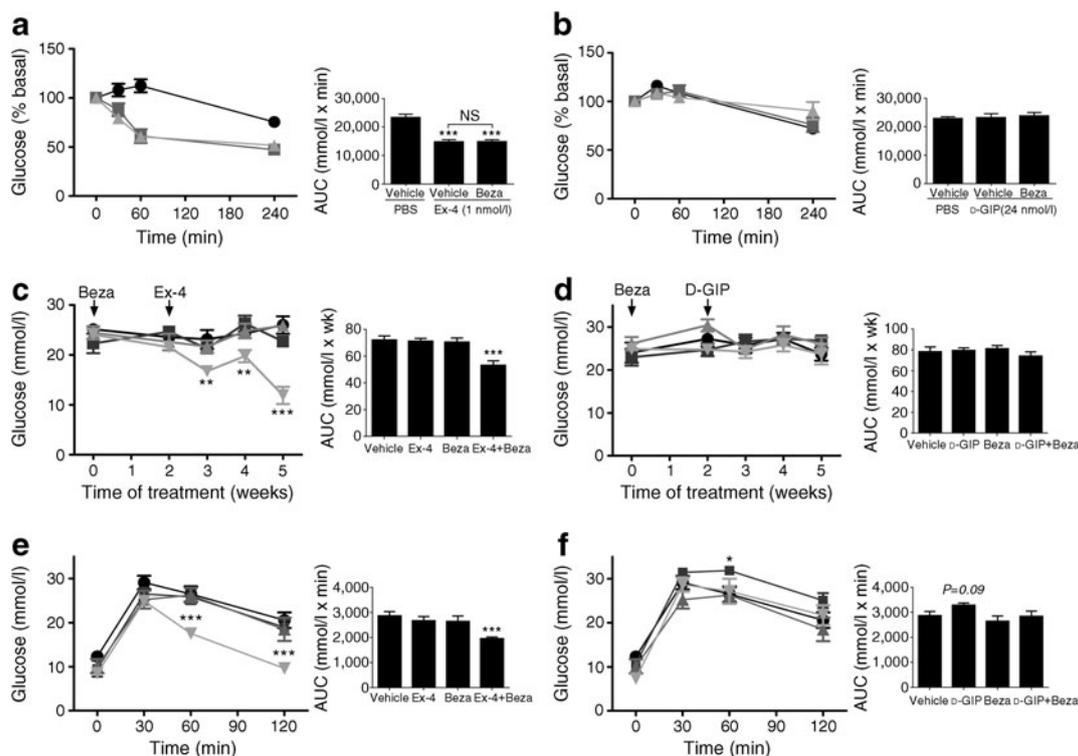


Fig. 6 Lipid lowering enhances the efficacy of exendin-4 (1 nmol/l), but not of D-GIP (24 nmol/l) in *db/db* mice. (a) The acute glucose-lowering effects of exendin-4 (Ex-4) or (b) D-GIP were assessed in *db/db* mice that had been orally treated with bezafibrate (Beza) or vehicle for 2 weeks. (a) Black circles, vehicle + PBS; grey squares, vehicle + Ex-4; grey triangles, Beza + Ex-4; (b) black circles, vehicle + PBS; grey squares, vehicle + D-GIP; grey triangles Beza + D-GIP. Following the pretreatment period, *db/db* mice were treated with additional exendin-4 (c) or D-GIP (d) for 3 weeks and random fed blood glucose levels measured once a week. (c) Black circles, vehicle; black squares,

Ex-4; upright triangles (grey), Beza; reversed triangles (grey), Ex-4 + Beza; (d) black circles, vehicle; black squares, D-GIP; upright triangles (grey), Beza; reversed triangles (grey), D-GIP + Beza. (e) OGTTs were performed in the Ex-4-Beza or (f) D-GIP-Beza groups after 2 weeks of co-treatment with bezafibrate and either exendin-4 or D-GIP. (e) Black circles, vehicle; black squares, Ex-4; upright triangles (grey), Beza; reversed triangles (grey), Ex-4 + Beza; (f) Black circles, vehicle; black squares, D-GIP; upright triangles (grey), Beza; reversed triangles (grey), D-GIP + Beza $n=5-8$ for each group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with vehicle groups

cholesterol levels, while bezafibrate alone or in combination with exendin-4 or D-GIP lowered serum triacylglycerol and NEFA levels, and increased serum total- and HDL-cholesterol levels (ESM Fig. 5).

Finally, we analysed the islet morphology of *db/db* mice after the various treatments using immunohistochemistry. As seen in Fig. 7a, islets from bezafibrate-, exendin-4- or D-GIP-treated *db/db* mice displayed a disordered islet morphology, which was similar to that in vehicle-treated *db/db* mice. Similarly, *db/db* mice treated with a combination of D-GIP and bezafibrate did not show any improvements in islet architecture compared with the vehicle group (Fig. 7a). However, mice treated with a combination of exendin-4 and bezafibrate displayed normalised islet architecture, with an increased proportion of beta cells, as well as normal distribution of alpha cells (Fig. 7a). Furthermore, only the exendin-4 plus bezafibrate group exhibited significantly increased beta cell mass and ratios of beta cell area to islet area compared with the vehicle-treated group (Fig. 7b, c). BrdU staining also showed that only the exendin-4 plus bezafibrate group significantly increased the number of BrdU-positive beta cells in islets compared with the vehicle-treated group (Fig. 7d, e). These data indicate that lipid lowering selectively enhanced the efficacy of GLP1R

but not GIPR agonists to improve islet morphology and glucose homeostasis, possibly through increases in pancreatic beta cell proliferation in *db/db* mice.

Discussion

Impaired incretin effects are found in type 2 diabetes [3, 4]. Our study was designed to investigate the role of hyperlipidaemia in the impairment of the incretin response in vitro and in vivo. In the in vitro models, we found that exposure to palmitate was sufficient for impairment of GLP1R, but not GIPR signalling to occur. This was evidenced by a reduced ability of GLP-1 to stimulate cAMP production, p-CREB and insulin secretion. The specificity of these defects was demonstrated by the partial restoration of signalling after Ad-GLP1R-mediated expression of exogenous *Glp1r*. In the in vivo models, we found that hyperlipidaemia was necessary for the downregulation of incretin receptor expression in islets of a mouse model of diabetes. Furthermore, in the *db/db* mouse model of diabetes, normalisation of the lipid profile by bezafibrate dramatically improved the efficacy of incretin-based therapies, including the DPP-4 inhibitor, des-fluoro-

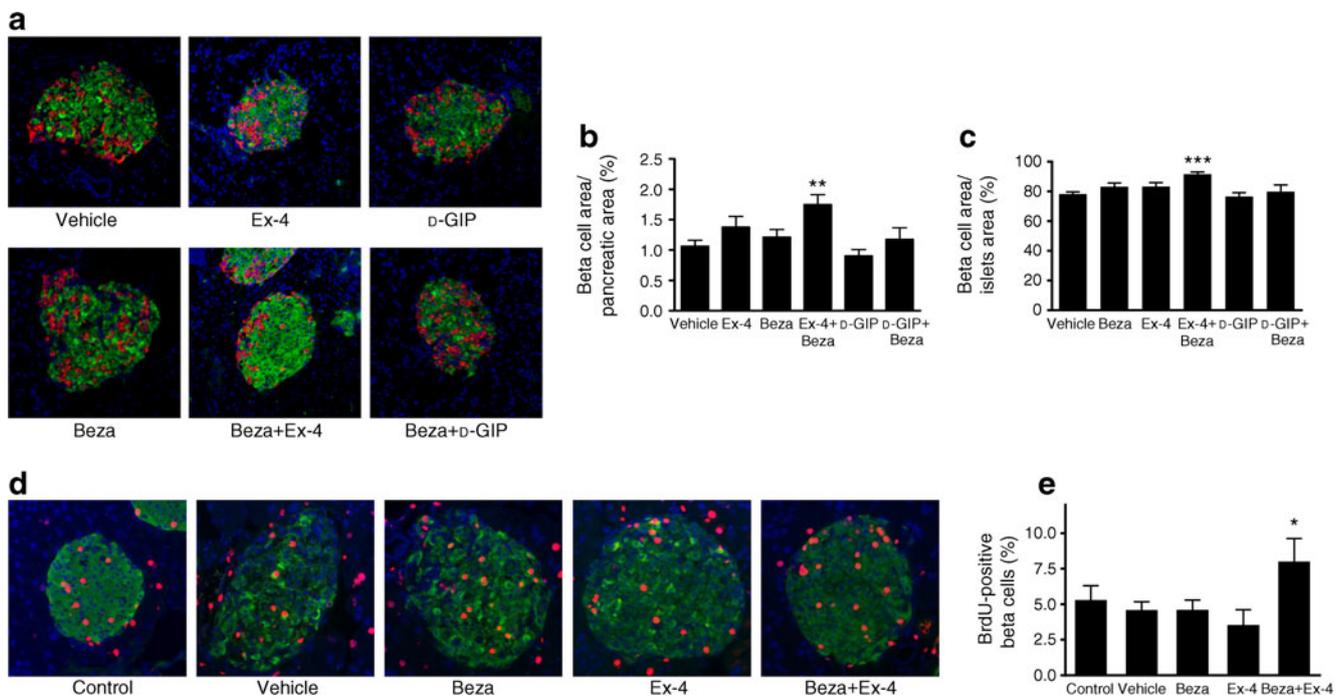


Fig. 7 Immunohistochemical analysis of the pancreas of *db/db* mice co-treated with bezafibrate (Beza) and either exendin-4 (Ex-4) or D-GIP. *db/db* mice were treated as in Fig. 7. (a) Pancreas was fixed in 4% paraformaldehyde and paraffin-embedded 4 μ m sections subjected to immunofluorescence analysis. Green, insulin; red, glucagon; blue, DAPI. (b) Beta cell mass expressed as the percentage of insulin-positive area to total pancreas area and (c) ratios of insulin-positive beta cell area to total islet area were assessed from staining (a) for

insulin and glucagon; $n=5-8$ each group; ** $p<0.01$ and *** $p<0.001$ compared with vehicle group. (d, e) Mice (*db/db*) were pretreated for 2 weeks with either bezafibrate or vehicle, and then treated with additional exendin-4 and BrdU (1 g/l in drinking water) for 10 days. Representative images (d) of pancreas sections stained for BrdU (red) and insulin (green) are shown. (e) BrdU-positive beta cells were quantified and expressed as percentage of respective islet beta cells; $n=3-4$ each group; * $p<0.05$ compared with vehicle group

sitagliptin, and the GLP1R agonist, exendin-4. These findings, together with the work of others [34], indicate crucial roles of fatty acids and GLP1R in maintaining incretin signalling, beta cell function and glucose homeostasis.

We and others have reported that GLP1R and GIPR levels were decreased in islets from mouse and rat models of diabetes, and from type 2 diabetic patients [27, 30, 35]. In the present study, we found that in vitro palmitate treatment resulted in reduced GLP1R levels. In islets isolated from *db/db* mice, we also observed a significant reduction of *Glp1r* expression. Furthermore, treatment of *db/db* mice with bezafibrate for 2 weeks, which significantly improved the serum lipid profile, partially restored *Glp1r* expression in islets, even though the hyperglycaemic status remained. These findings imply that, apart from hyperglycaemia, hyperlipidaemia is required for downregulation of *Glp1r* expression in diabetes. GIPR was less sensitive to regulation by palmitate. This difference in the regulation of incretin receptors by fatty acids is reminiscent of the effects of hyperglycaemia; thus conscious rats receiving glucose infusions and isolated rat islets exposed to high glucose exhibited decreases in *Glp1r* but not *Gipr* expression [27]. Although *Gipr* expression was unaltered in palmitate-treated INS-1E cells, GIP-stimulated cAMP production and insulin secretion were significantly decreased. This discrepancy in findings for GIP is possibly due to NEFA-induced global beta cell dysfunction via other pathways involving endoplasmic reticulum stress, oxidative stress and Ca^{2+} homeostasis [29, 31]. For example, SREBP1c, which was reported to mediate palmitate-induced impairment of insulin secretion in islets [36], was increased in INS-1E, but not in MIN6 cells after palmitate treatment. The results probably reflect the complexity of the effects of hyperlipidaemia on beta cell function, with impaired incretin receptor signalling contributing to beta cell glucolipotoxicity in concert with other pathways involving endoplasmic reticulum and oxidative stress [29, 31].

Although the glucose-lowering efficacy of incretin agonists and DPP-4 inhibitors has been shown in animal models [37–39], it is worth noting that chronic treatment of *db/db* mice with incretin agonists or DPP-4 inhibitors alone only delays the onset of diabetes at the early stages of disease progression [40]. Exendin-4 treatment does not prevent the ongoing deterioration of glucose intolerance in severely diabetic *db/db* mice [41]. Likewise, clinical evidence shows that the efficacy of incretin-based drugs for the treatment of type 2 diabetes is variable, and may be affected by various factors such as age [42], stage and severity of diabetes, differences in responsiveness to GLP-1 in diverse ethnic groups, genetic variance of GIPR and GLP1R [43, 44], as well as hyperglycaemia [27]. The current study demonstrates for the first time that hyperlipidaemia should be included as a contributing factor to the reduced efficacy of incretin-based drugs in mouse models of diabetes.

Hyperlipidaemia is closely associated with type 2 diabetes, and glucose-lowering drugs such as thiazolidinediones and metformin improve glucose and lipid metabolism [45]. Our in vitro data show that elevated NEFA is sufficient to cause impaired GLP1R signalling, prompting us to test the relationship between hyperlipidaemia and the efficacy of incretin-based therapy in animal models of diabetes. The lipid-lowering agent bezafibrate significantly improved the serum lipid profile, without affecting blood glucose levels in *db/db* mice. Strikingly, after lipid lowering, the DPP-4 inhibitor des-fluoro-sitagliptin and the GLP1R agonist exendin-4 both had a more robust effect on glycaemic control than co-treatment with vehicle or treatment with each agent alone. The effects were not due to increases in insulin sensitivity. Rather the improved glucose tolerance was associated with restoration of normal islet morphology and increased beta cell mass. This effect was only apparent with prolonged incretin activation, since the lowering of fatty acids did not enhance glucose disappearance after acute treatment of *db/db* mice with exendin-4. These results suggest that the improved glucose homeostasis induced by chronic administration of exendin-4 and bezafibrate to *db/db* mice is effected via long-term improvements in beta cell mass and function, which may be due to restored expression of *Glp1r* and thus GLP1R signalling after lipid lowering. However, although *Gipr* expression was also partially restored by bezafibrate treatment, the GIPR agonist D-GIP did not improve glucose metabolism. Recent reports have demonstrated that GLP1R signalling exerts more robust control of beta cell survival and regeneration than does GIPR signalling in mice [46]. It has also been reported that GLP-1-stimulated p-CREB plays important roles in the regulation of beta cell survival through GLP1R activation [33]. In this study, we only observed reduced GLP-1-stimulated p-CREB during in vitro palmitate treatment. Moreover, only combined treatment with exendin-4 plus bezafibrate improved islet morphology and increased beta cell mass, associated with increased beta cell proliferation in *db/db* mice. On the other hand, it has been reported that GIP was associated with impaired insulin sensitivity [38, 47], and this may partially explain the differences in efficacy of the incretin receptor agonists.

The peroxisome proliferator-activated receptor (PPAR)- α agonist WY14643 has been reported to increase *Gipr* expression [48]; at the same time, PPAR- α activation is associated with improved beta cell survival and function through reduction of lipid accumulation by increased fatty acid oxidation in beta cells and human islets [49, 50]. It has also been reported that metformin increases *Glp1r* expression in INS-1 cells via a PPAR- α -dependent pathway [51]. Our results demonstrate that palmitate downregulates GLP1R in insulinoma cell lines and bezafibrate can improve the efficacy of des-fluoro-sitagliptin and exendin-4 in mouse

models of diabetes. Intriguingly, the related nuclear receptor PPAR- γ has been reported to regulate *Gipr* expression by binding to the PPAR response elements within the rat *Gipr* promoter [52]. The question of whether PPAR- α activation regulates incretin receptors in beta cells through direct binding or indirect improvement in lipotoxicity requires further investigation.

In summary, our results show that hyperlipidaemia contributes to impaired beta cell responsiveness to GLP-1, partially through downregulation of GLP1R. Combined treatment with incretin-based drugs (des-fluoro-sitagliptin or exendin-4) and lipid-lowering drugs (bezafibrate) results in synergistic improvements of glucose metabolism and islet morphology and function. These findings reinforce the importance of lipid management in type 2 diabetes and provide important information for the design of new incretin-based therapies for the treatment of type 2 diabetes mellitus.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement ZK, RF, YZ, JL and DRL performed experiments, analysed the data and contributed to the revision of the article. YD and JC contributed to the acquisition of data and revision of the article. ZK, YD and DRL analysed the data and wrote the manuscript. GX conceived and designed the experiments, performed experiments and wrote the manuscript. All authors approved the final version of the article.

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