The handle http://hdl.handle.net/1887/32608 holds various files of this Leiden University dissertation.

**Author:** Ellenbroek, Johanne Hendrike (Rianne)
**Title:** Pancreatic $\beta$- and $\alpha$-cell adaptation in response to metabolic changes
**Issue Date:** 2015-25-03
Chapter 5

Glucagon like peptide-1 receptor agonist treatment reduces β-cell mass in normoglycaemic mice

Johanne H. Ellenbroek1, Hendrica A. M. Töns1, Menso J. A. Westerouen van Meeteren1, Natascha de Graaf1, Maaike A. Hanegraaf1, Ton J. Rabelink1, Françoise Carlotti1, Eelco J. P. de Koning1,2

1Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands,
2Hubrecht Institute, Utrecht, The Netherlands

Diabetologia 2013, 56: 1980-6
Abstract

Aims/hypothesis
Incretin-based therapies improve glycaemic control in patients with type 2 diabetes. In animal models of diabetes, glucagon-like peptide-1 receptor agonists (GLP-1RAs) increase β-cell mass. GLP-1RAs are also evaluated in non-diabetic individuals with obesity and cardiovascular disease. However, their effect on β-cell mass in normoglycaemic conditions is not clear. Here, we investigate the effects of the GLP-1RA liraglutide on β-cell mass and function in normoglycaemic mice.

Methods
C57BL/6J mice were treated with the GLP-1RA liraglutide or PBS and fed a control or high-fat diet (HFD) for 1 or 6 weeks. Glucose and insulin tolerance tests were performed after 6 weeks. BrdU was given to label proliferating cells 1 week before the animals were killed. The pancreas was taken for either histology or islet isolation followed by a glucose-induced insulin-secretion test.

Results
Treatment with liraglutide for 6 weeks led to increased insulin sensitivity and attenuation of HFD-induced insulin resistance. A reduction in β-cell mass was observed in liraglutide-treated control and HFD-fed mice at 6 weeks, and was associated with a lower β-cell proliferation rate after 1 week of treatment. A similar reduction in α-cell mass occurred, resulting in an unchanged α- to β-cell ratio. In contrast, acinar cell proliferation was increased. Finally, islets isolated from liraglutide-treated control mice had enhanced glucose-induced insulin secretion.

Conclusions/interpretation
Our data show that GLP-1RA treatment in normoglycaemic mice leads to increases in insulin sensitivity and β-cell function that are associated with reduced β-cell mass to maintain normoglycaemia.
Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted by intestinal L cells in response to ingestion of carbohydrates and lipids (1). Activation of the GLP-1 receptor (GLP-1R) on pancreatic β-cells leads to glucose-dependent insulin secretion and improves glycaemic control in patients with type 2 diabetes (2, 3). In animal models of diabetes these therapies increase β-cell mass (4–6).

The β-cell mass is tightly controlled in order to keep glucose levels within a narrow range. When the demand for insulin is chronically increased by physiological or pathological changes, such as pregnancy or obesity, there is an increase in β-cell function and/or β-cell mass (7, 8). When the demand for insulin decreases, for example postpartum, the β-cell mass reverts to its original capacity (9).

Besides their effects on β-cells, GLP-1R agonists (GLP-1RAs) exert several extrapancreatic effects that may be of therapeutic benefit. GLP-1RAs decrease body weight and are associated with reduced blood pressure, improved lipid profiles and improved endothelial function in patients with type 2 diabetes (10, 11). Therefore, these compounds have also been evaluated in non-diabetic individuals with obesity or cardiovascular disease (12–15). However, the effect of GLP-1RAs on β-cells in these normoglycaemic conditions is not clear. Therefore, we investigated the effects of GLP-1RA treatment on β-cell mass and function in normoglycaemic mice.

Methods

Animals

Male C57BL/6J mice, 8–9 weeks old (Charles River Laboratories, Wilmington, MA, USA), were housed under standard conditions with a 12 h light/dark cycle and free access to food and water. Mice were fed a normal diet (control; 10% of total energy intake derived from lard fat, 16.3 kJ [3.9 kcal]/g; D12450B, Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD; 45% of total energy intake derived from lard fat, 19.7 kJ [4.7 kcal]/g; D12451, Research Diets) for 1 or 6 weeks. Average food intake was determined weekly per cage housing three or four mice. Liraglutide (0.1 mg/kg, Novo Nordisk, Bagsvaerd, Denmark) or PBS was given twice daily with at least a 10 h interval between subcutaneous injections. Treatment was discontinued 1 day before the animals were killed. Body weight was determined after overnight fasting. Animal experiments were approved by the institutional ethical committee on animal care and experimentation at the Leiden University Medical Center.
Glucose and insulin tolerance tests
Insulin tolerance and glucose tolerance were assessed after 6 weeks of treatment. An intraperitoneal insulin tolerance test (ITT) was performed in animals that had been fasted for 6 h. After measuring basal blood glucose concentration from the tail vein, 0.75 U/kg insulin was injected followed by monitoring of the blood glucose concentrations after 15, 30 and 60 min. An intraperitoneal glucose tolerance test (GTT) was performed in overnight-fasted mice. Blood samples were drawn from the tail vein before injecting 2 g/kg glucose and after 15, 30, 60 and 120 min. Blood glucose concentrations were measured using a glucose meter (Accu-Chek, Roche, Basel, Switzerland) and insulin concentrations were measured by ELISA (Chrysal Chem, Downers Grove, IL, USA). Plasma IL-6, IL-1β and monocyte chemoattractant protein-1 (MCP-1) were detected using a custom cytokine/metabolic multiplex assay (Meso Scale Discovery, Gaithersburg, MD, USA).

Pancreas dissection and islet isolation
Mice were anaesthetised by isoflurane inhalation and exsanguinated. The pancreases were dissected and weighed. For immunohistochemistry the pancreas was fixed by immersion in a 4% (vol./vol.) paraformaldehyde solution. For islet isolation the pancreases of six to eight mice were pooled and digested using 3 mg/ml collagenase (Sigma-Aldrich, St Louis, CA, USA) in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 μg/ml DNAse I (Pulmozyme, Roche) and shaken at 37°C for 15–18 min until a homogeneous digest was obtained. The digest was then washed three times with cold RPMI medium supplemented with 10% (vol./vol.) heat-inactivated FCS (Bodinco, Alkmaar, the Netherlands) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively; Invitrogen). Islets were purified from exocrine tissue by manual selection picking under a dissecting microscope.

Glucose-induced insulin secretion test
A glucose-induced insulin secretion test was performed on freshly isolated islets. Groups of ten islets were incubated in a modified Krebs-Ringer bicarbonate buffer with HEPES (KRHB) containing 115 mmol/l NaCl, 5 mmol/l KCl, 24 mmol/l NaHCO3, 2.2 mmol/l CaCl2, 1 mmol/l MgCl2, 20 mmol/l HEPES and 2 g/l human serum albumin (Caelb, Sanquin, the Netherlands), pH 7.4. Islets were washed and pre-incubated with KRHB buffer containing 2 mmol/l glucose for 1.5 h at 37°C. They were then incubated in 2 mmol/l glucose KRHB buffer for 1 h at 37°C and switched to 20 mmol/l glucose KRHB buffer for 1 h at 37°C. Supernatant fractions were kept for determination of insulin concentration by ELISA (Mercodia, Uppsala, Sweden). Islet cells were lysed by sonication in distilled water. Islet insulin content was measured by acid ethanol extraction followed by ELISA (Mercodia). Islet DNA content was determined by Quant-iT PicoGreen dsDNA kit (Invitrogen).
Immunohistochemistry and morphometry

In order to obtain representative samples of the entire organ, pancreases from each mouse (six per group) were cut into three pieces (duodenal, gastric and splenic region) that were embedded in paraffin blocks and sliced into 4 μm sections. For each analysis two to four sections per block, with an interval of at least 200 μm between sections, were immunostained and analysed. The average of the three regions was taken as a measure for the entire organ.

For the identification of β-cells, nine to twelve sections per mouse pancreas were immunostained with guinea pig anti-insulin IgG (Millipore, Billerica, MA, USA) or rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h followed by horseradish peroxidase (HRP)- or alkaline phosphatase-conjugated secondary antibodies for 1 h. α-Cells were identified by immunostaining by rabbit anti-glucagon IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h followed by HRP-conjugated secondary antibody for 1 h. Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) or Liquid Permanent Red (LPR; Dako, Glostrup, Denmark) and counterstained with haematoxylin. Stained sections were digitally imaged (Panoramic MIDI, 3DHISTECH, Budapest, Hungary).

β-cell and pancreas areas stained with haematoxylin were determined using an image-analysis program (Stacks 2.1, LUMC, Leiden, the Netherlands), excluding large blood vessels, larger ducts, adipose tissue and lymph nodes as previously described. β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster area was determined as the average area of β-cell clusters (defined as ≥4 β-cells per cluster) per mouse. α-Cell mass was determined by calculating the ratio of α-cell area per β-cell area per islet, using ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD, USA), multiplied by the β-cell mass.

To label proliferating β-cells, mice were given 50 mg/kg BrdU (Sigma-Aldrich) subcutaneously twice daily during the entire period for the 1 week study and the final 7 days for the 6 week study. Sections were double stained for insulin-LPR and BrdU (BrdU staining kit, Invitrogen). Stained sections were digitally imaged (Panoramic MIDI). BrdU-positive β-cells were assessed as a proportion of all β-cells. Pancreatic duct cells were identified based on their typical morphology and location. The number of BrdU-positive duct cells was counted. The number of BrdU-positive acinar cells was counted using Stacks 2.1 and expressed as a percentage of the total number of acinar cells. The area in which these were counted was divided by the total number of cells as a measure of acinar cell size. Apoptotic β-cells were identified by the TUNEL technique (Roche) in combination with insulin immunostaining and were counted. The investigator was blind to the experimental conditions during counting.
**Statistical analysis**
Data are presented as mean±SEM. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by two-way ANOVA, followed by Bonferroni’s multiple comparisons test, as appropriate. $p<0.05$ was considered statistically significant.

**Results**

**Metabolic characteristics of control and HFD-fed mice following 6 weeks of liraglutide treatment**
Liraglutide treatment for 6 weeks was associated with decreased body weight and increased insulin sensitivity in both control and HFD-fed mice (Fig. 1a–c). After a glucose load, liraglutide treatment attenuated the peak glucose concentration induced by HFD at 30 min (Fig. 1d), but this did not reach significance for the AUC for glucose (Fig. 1e). In mice on a normal diet there was no significant difference in glucose concentrations after liraglutide treatment (Fig. 1d). In both liraglutide-treated groups the early response of insulin secretion during the GTT was increased to a similar extent as in HFD-fed mice (Fig. 1f, g).

**Increased acinar cell proliferation after liraglutide treatment**
After 6 weeks, pancreatic weight was significantly greater in liraglutide-treated mice (Fig. 2a), despite the decrease in body weight (Fig. 1a). Therefore, we analysed the effect of liraglutide treatment on the exocrine pancreas. Treatment with liraglutide was associated with an increased size of acinar cells in control mice (Fig. 2b). We counted 13,441±439 acinar cells per mouse. The number of proliferating acinar cells was 65% higher in liraglutide-treated control mice (Fig. 2c, d). A similar effect was observed in liraglutide-treated HFD-fed mice, though the difference was less prominent. No significant difference in the number of proliferating duct cells was observed between the groups after 6 weeks of treatment (528±28 duct cells were counted per mouse; Fig. 2e, f). Assessment of pro-inflammatory cytokine plasma concentrations (IL-1β, IL-6 and MCP-1) showed no significant differences between the groups (electronic supplementary material [ESM] Fig. 1).
**Fig. 1.** Metabolic characteristics of control (squares) and HFD-fed (circles) mice treated with liraglutide (black) or PBS (white) for 6 weeks. (a) Body weight (n = 13–14 mice). (b) Blood glucose concentrations expressed as percentage of basal glucose concentration during the ITT (n = 7–8 mice). (c) AUC of glucose concentrations during the ITT corrected for basal glucose concentration (n = 7–8 mice). (d) Blood glucose concentrations during GTT (n = 6 mice). (e) AUC for blood glucose concentrations during the GTT (n = 6 mice). (f) Insulin concentrations during GTT (n = 5–6 mice). (g) AUC 0–15 min insulin concentrations during GTT (n = 5–6 mice). *p < 0.05, **p < 0.01 and ***p < 0.001; † p < 0.05 vs control + PBS; ‡ p < 0.05 vs control + PBS and HFD + liraglutide.
Fig. 2. The effects of liraglutide (black bars) or PBS (white bars) treatment on the exocrine pancreas in control and HFD-fed mice after 6 weeks. (a) Pancreas weight (n=13–14 mice). (b) Acinar cell size (n=6 mice). (c) Representative picture of proliferating acinar cells, BrdU (brown); scale bar, 100 μm. (d) Acinar cell proliferation, BrdU labelling during the final 7 days (n=6 mice). (e) Duct cell proliferation, BrdU labelling during the final 7 days (n=6 mice). (f) Representative picture of proliferating duct cells (arrows), BrdU (brown); scale bar, 50 μm. *p<0.05, **p<0.01 and ***p<0.001.

**Liraglutide reduces β-cell and α-cell mass**

β-cell mass, determined by analysing 94.9 ± 4.7 mm² pancreatic tissue per mouse, was reduced in control and HFD-fed mice after treatment with liraglutide for 6 weeks (Fig. 3a). This was associated with a decreased average β-cell cluster area (Fig. 3b). Liraglutide treatment did not affect insulin content in freshly isolated islets (Fig. 3c). No difference in the number of apoptotic β-cells was found between groups after counting 1,129 ± 170 β-cells per mouse (Fig. 3d). To determine the number of proliferating β-cells 1,807 ± 128 cells per mouse were counted. Liraglutide treatment in both control and HFD-fed mice was associated with a lower number of proliferating β-cells after 1 week, but after 6 weeks no difference was observed (Fig. 3e–g). Similarly, the α-cell mass was reduced in liraglutide-treated control mice. The lower α-cell mass in HFD-fed mice was unaffected by liraglutide treatment (Fig. 3h). The ratio of α- to β-cell area did not change significantly between the groups (Fig. 3i).
GLP-1RA treatment reduces β-cell mass in normoglycaemic mice

**Fig. 3.** β-Cell and α-cell mass in control and HFD-fed mice after liraglutide (black bars) or PBS (white bars) treatment. (a) β-Cell mass after 6 weeks of treatment (n=6 mice). (b) β-Cell cluster area after 6 weeks of treatment (n=6 mice). (c) β-cells Insulin content from isolated islets corrected for DNA content (n=24). (d) β-Cell apoptosis, identified by TUNEL+insulin+ staining, after 6 weeks of treatment (n=6). (e) β-Cell proliferation in control mice after 1 and 6 weeks of treatment, BrdU labelling during the final 7 days (n=6 mice). (f) β-Cell proliferation in HFD-fed mice after 1 and 6 weeks of treatment, BrdU labelling during the final 7 days (n=6 mice). (g) Representative picture of proliferating β-cells (arrows), BrdU (brown) and insulin (red). Scale bar=50 μm. (h) α-Cell mass after 6 weeks of treatment (n=6 mice). (i) Ratio of α-cell area to β-cell area after 6 weeks of treatment (n=6 mice). *p<0.05, **p<0.01 and ***p<0.001.

**Increased glucose-induced insulin release from isolated islets of liraglutide-treated control mice**

Finally, in order to investigate whether 6 weeks of liraglutide treatment had specific effects on β-cell function in the presence of a reduced β-cell mass, we assessed glucose-induced insulin secretion in isolated islets. Basal insulin secretion (2 mmol/l glucose) was increased twofold in islets from liraglutide-treated control mice (Fig. 4a). Glucose stimulation of islets from liraglutide-treated control mice resulted in a 35% increase in insulin secretion (Fig. 4a). As expected, glucose stimulation of islets from HFD-fed mice led to increased insulin secretion compared with mice fed...
regular chow (Fig. 4b). This glucose stimulation was unchanged in HFD-fed mice that had been treated with liraglutide for 6 weeks (Fig. 4b).

![Fig. 4](image)

**Fig. 4.** Glucose-induced insulin secretion from isolated islets of control mice and HFD-fed mice treated with liraglutide for 6 weeks. Insulin secretion is presented as a percentage of total insulin content. (a) Insulin secretion of islets from control mice, \( n = 24 \), and (b) insulin secretion of islets from HFD-fed mice, \( n = 23 - 24 \); white bars, 2 mmol/l glucose; grey bars, 20 mmol/l glucose. *\( p < 0.05 \), **\( p < 0.01 \) and ***\( p < 0.001 \).

### Discussion

The effect of GLP-1-based therapy on insulin secretion from \( \beta \)-cells has been reported to be glucose dependent (17–19), but its effect on \( \beta \)-cell mass under different glycaemic conditions is less clear. While GLP-1RA treatment increases \( \beta \)-cell mass in animal models of diabetes, we now show a reduction in \( \beta \)-cell mass in normoglycaemic mice. GLP1-RA treatment of non-diabetic obese individuals results in weight loss and improved \( \beta \)-cell function (12, 13). Therefore, it is relevant to understand how \( \beta \)-cell mass adapts during GLP1-RA treatment under normoglycaemic conditions and different dietary situations. So far, few studies have investigated the effect of GLP-1RA in non-diabetic animals and showed either no difference or an increase in \( \beta \)-cell proliferation after short-term treatment for between 2 and 10 days (6, 20–22).

Liraglutide treatment for 6 weeks resulted in decreased body weight and increased insulin sensitivity in normoglycaemic mice and HFD-fed mice, in line with earlier studies (23, 24). In addition, the increased early response of insulin secretion during the GTT in liraglutide-treated mice is in line with the working mechanism of GLP-1 (25, 26). This was associated with a major reduction in \( \beta \)-cell mass in both control and HFD-fed mice. The lower \( \beta \)-cell proliferation rate that we observed in mice treated for 1 week suggests that the \( \beta \)-cell mass adapted rapidly after the start of liraglutide treatment. Interestingly, we also show that \( \alpha \)-cell mass was reduced to such an extent that the ratio of \( \alpha \)- to \( \beta \)-cells remained unchanged.
Acute GLP-1R stimulation of β-cells is known to increase insulin secretion in a glucose-dependent manner (17–19). We show that sustained GLP-1RA treatment during normoglycaemic conditions is associated with increased insulin secretion from isolated islets even in the absence of direct GLP-1RA stimulation in vitro. In HFD-fed mice, liraglutide treatment increased insulin sensitivity, but the enhanced insulin secretory response remained. Together these data suggest that liraglutide treatment in normoglycaemic mice leads to increased insulin sensitivity and an enhanced insulin secretory response from existing β-cells, thereby reducing the need for new β-cells. In addition, these data imply that chronic GLP-1RA treatment during normoglycaemia results in an increased β-cell function as was shown in non-diabetic obese individuals (12). In contrast, GLP-1RA treatment does not increase β-cell proliferation during normoglycaemia.

Finally, we observed increased acinar cell proliferation after liraglutide treatment indicating that this effect can occur during normoglycaemia. There was a non-significant difference in duct cell proliferation after 6 weeks of treatment. These observations and the findings of other studies (27–32) raise the issue of whether GLP-1-based therapies are a potential risk for pancreatitis; some studies did not observe this effect (33, 34), however, which may reflect the animal model used, the age of the animals and/or the labelling and counting methods used for proliferating cells. In our study, there was no indication of a systemic inflammatory state. Ductal proliferation is also associated with the development of pancreatic adenocarcinoma but the relationship between GLP-1-based treatment and the development of new pancreatic malignant lesions is not clear (35).

In conclusion, our data indicate that GLP-1RA under normoglycaemic conditions can have different effects on pancreatic islet and non-islet cells. GLP-1RA treatment during normoglycaemia results in a reduction in β-cell mass, whereas it exerts proliferative effects on the exocrine pancreas.
Supplementary material

**ESM Fig. 1.** The effect of liraglutide (black bars) or PBS (white bars) treatment on pro-inflammatory cytokines after 6 weeks. (a) Plasma interleukin-1β (IL-1β) concentrations. (b) Plasma monocyte chemoattractant protein-1 (MCP-1) concentrations. (c) Plasma interleukin-6 (IL-6) concentrations. HFD = high-fat diet.
GLP-1 RA treatment reduces β-cell mass in normoglycaemic mice

References


