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Liraglutide, but not vildagliptin, restores normoglycaemia and insulin content in the animal model of type 2 diabetes, *Psammomys obesus*

Louise Vedtofte [a,1], Thóra B. Bodvarsdottir [b,1], Carsten F. Gotfredsen [b], Allan E. Karlsen [b], Lotte B. Knudsen [b], R. Scott Heller [a,*]

[a] Department of Beta Cell Regeneration, Hagedorn Research Institute, Gentofte, Denmark
[b] Diabetes Research Unit, Novo Nordisk A/S, Maaloev, Denmark

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ABSTRACT

In order to investigate the effect and mechanism of liraglutide and vildagliptin in diabetic *Psammomys obesus*, we examined proliferation and apoptosis of β-cells, β-cell mass (BCM), and pancreatic insulin content after zero, six and fourteen days of treatment compared to control groups. One group of animals was kept on low-energy diet and seven groups were given high-energy diet (HED) that induced diabetes over a four week period. Non-fasting morning blood glucose, body weight, HbA_{1C} and pancreatic insulin content were measured and beta cell mass (BCM), proliferation and apoptosis frequencies were determined using stereological point counting. Liraglutide significantly reduced blood glucose and even normalized it in all animals treated for six days and in 11 out of 17 animals treated for fourteen days. HED increased BCM and treatment with liraglutide did not change this. However, compared to the vehicle-treated animals pancreatic insulin content was normalized in animals treated for six and fourteen days with liraglutide. In contrast, vildagliptin, in doses causing full inhibition of plasma DPP-IV activity, neither reduced blood glucose nor altered HED-induced increases in BCM or pancreatic insulin content. These results suggest that liraglutide restores normoglycaemia and improves glycaemic control in *P. obesus* by increasing their insulin content and improving the function of the β-cells. In contrast, vildagliptin does not improve glycaemic control in *P. obesus* nor affect β-cell insulin content.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the result of progressive loss and dysfunction of pancreatic β-cells [1]. It can begin at birth if exposed to growth retardation or result from genetic mutations, i.e. the MODY variants, or later in life where obesity and subsequent insulin resistance and β-cell deterioration results in diabetes [2]. β-cell function is crucial for glycaemic control and the reduced functional β-cell mass (BCM) is critical for the development of glucose intolerance, hyperglycaemia, and late-diabetic complications. *P. obesus* is a well-established outbred model of T2DM that when kept on a high-energy diet (HED) develops diabetes [3]. This begins with hyperinsulinaemia, followed by hyperglycaemia, and finally overt T2DM occurs. The progression can be divided into four stages [4]: Stage A is characterized by normoglycaemia and normoinsulinaemia; stage B is characterized by normoglycaemia and hyperinsulinaemia, and stage C is characterized by hyperglycaemia and hyperinsulinaemia. Both stages B and C are associated with prominent peripheral and hepatic insulin resistance without suppression of gluconeogenesis. Additionally, hepatic and adipose tissue lipogenesis is increased. This effect on sustained hepatic lipogenesis is in contrast to what is seen in other rodent models, but is in common with humans, where the liver is the primary site of lipogenesis. Both stages B and C are reversible in calorie restricted animals. The last stage D is an irreversible stage of hyperglycaemia and insulinopenia. These stages of type 2 diabetes development closely resemble the phenotypic pattern observed in humans, making *P. obesus* in stage B and C a good animal model for the study of intervention against, or reversal of, nutrition-induced diabetes, whereas stage D is suitable for insulin replacement therapy studies [5–8].

Glucagon-like peptide-1 (GLP-1) is a peptide hormone, with a very short half-life, which is secreted from the L-cells in the lower gut in response to food intake [9,10] and is able to increase insulin secretion and lower blood glucose and glucagon secretion in diabetic patients [11–14]. Liraglutide is a GLP-1 analogue with 97% amino acid homology to human GLP-1 with a fatty acid attached. This causes non-covalent binding to albumin and protects it from degradation by DPP-IV and reduces renal clearance which increases plasma half-life to ~13 h after subcutaneous injection in man [15]. Several studies

* Corresponding author. Tel.: +45 44 43 9137; fax: +45 44 43 8000.
E-mail address: shll@hagedorn.dk (R.S. Heller).
[1] These authors contributed equally and should be considered co-first authors.

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have demonstrated the potent effects of liraglutide on glycaemic control in diabetic animals and T2DM patients [16–18]. Additionally, GLP-1 and GLP-1 receptor agonists have been shown to increase proliferation, neogenesis, BCM, insulin biosynthesis, and insulin content and decrease apoptosis in different animal models [19–28].

Vildagliptin (LAF237) is a small molecule that blocks the action of DPP-IV and thereby prolongs the action of GLP-1, glucose-dependent insulinotropic peptide (GIP), and other peptides [29]. Vildagliptin is administered orally and has a half-life of 2–3 h in humans [30] and has been shown to improve glycaemic control in humans [20,31] and animals [32–35]. Additionally, vildagliptin, or other DPP-IV inhibitors, have been shown to increase proliferation, neogenesis, BCM, and pancreatic insulin content, and decrease apoptosis in rodents [36–39].

We have previously shown, that liraglutide is able to reduce blood glucose in *P. obesus* but the mechanisms are unknown [40]. Hence, the objective of the present study was to elude these mechanisms in diabetic *P. obesus*. In order to investigate if the normalization could be explained by an increase in BCM or improved insulin content, we examined by stereological methods β-cell proliferation, apoptosis, and neogenesis as well as measured pancreatic insulin content. Furthermore, we studied to what extend a DPP-IV inhibitor, vildagliptin, would be able to normalize blood glucose and influence the same parameters as listed above, as the effects of a DPP-IV inhibitor has never been investigated in *P. obesus*.

2. Methods

2.1. Formulation of liraglutide

Two ml of 6.25 mg/ml liraglutide (batch no. PQ50366) was dissolved in 60.5 ml vehicle (Phosphate buffered saline, D-PBS, Gibco ref. 14190-0094) so the final concentration of liraglutide was 200 µg/ml. Liraglutide 200 µg/ml was filled in 3 ml NovoPens, closed with green tops and stored at 4 °C . Vials with vehicle solution were kept at 4 °C and 100 µl per animal was used for the vehicle treatment.

2.2. Formulation of vildagliptin

Vildagliptin, synthesized at Novo Nordisk, was weighed out and suspended in vehicle (0.6% Carboxy methyl cellulose (CMC) (high viscosity) and 0.2% $NaHCO_3$) to the final concentrations of 2.5, 7.5 and 15 mg/ml for 10, 30 and 60 mg/kg, respectively. The suspensions were stored at 4 °C for a maximum of one week.

2.3. Treatment with liraglutide or vildagliptin in P. obesus

Male and female *P. obesus*, aged 7–11 weeks, were obtained from Harlan (Jerusalem, Israel). Animals were housed three per cage under controlled conditions with a 12 h light:12 h dark cycle, at temperature of 25 °C and fed low-energy (LE-2.4 kcal/g) chow (Koffolk 19560, Harlan, Israel) and water *ad libitum*. The animals of both sexes were allowed 4–7 weeks of acclimatization and were 14–15 weeks old at the start of experiments. A control group of ten animals was sacrificed before transfer to high-energy diet.

At the age of 14–15 weeks, animals were transferred to an *ad libitum* HED (3.1 kcal/g-Purina 5008, Purina Mills, Gray Summit, MO) for 3–4 weeks during which body weight (BW), morning blood glucose (morning BG) and HbA_{1C} were measured three times weekly. The animals that developed hyperglycaemia, defined as morning BG above 10 mM on two consecutive readings, two days apart, were used in the treatment study and the others discarded from the study. Animals that were diabetic after 3–4 weeks of *ad libitum* HED were allocated into groups with the same mean morning BG and HbA_{1C}, measured the day before start of treatment and randomized. One group of animals (for pancreas histology, $n=6$) was sacrificed before start of treatment. In the dose–response studies with liraglutide or vildagliptin, vehicle or different doses of liraglutide (0.0125, 0.025, 0.05, 0.1, 0.15 or 0.3 mg/kg, $n=4$–7 animals) were administered subcutaneously (s.c.) once daily in the morning after measurement of morning BG. Vehicle and different doses of vildagliptin (10, 30, or 60 mg/kg, $n=4$–7 animals) were administered orally twice daily (at 7 and 19 h) for four weeks.

In the main study, vehicle (s.c. and oral), liraglutide (0.2 mg/kg/day s.c.) or vildagliptin (30 mg/kg/day orally) were administered twice daily for 6 or 14 days ($n=10$–17).

In all studies with liraglutide, except for the 6 days study, the dose was titrated up over two weeks from 0.0125 mg/kg in all dose groups and the dose volume was 1 ml/kg. This allowed for the maximum dose to be reached near the end of the 14 days. All treatment groups were kept on HED throughout the study and morning BG and BW were measured 1–3 times a week and HbA_{1C} once weekly.

At sacrifice, pancreata from selected groups were evaluated histologically for insulin content and BCM as described below and blood was collected to measure DPP-IV activity and intact GLP-1 levels. The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

2.4. Morning BG

Blood samples, for determination of whole blood glucose concentration, were taken from the tail tip capillary into 5 µl glass capillary tubes and immediately suspended in buffer (250 µl of EBIO analysis buffer) and analyzed for glucose on the test day. Glucose concentrations were analyzed by the immobilised glucose oxidase method using an EBIO Plus autoanalyzer as described by manufacturer (Eppendorf, Horsholm, Denmark).

2.5. HbA1c%

Blood for the determination of HbA_{1c} was collected into heparinized 5 µl glass tubes by puncture of the capillary vessel in the tail tip. Samples were immediately diluted in 250 µl analysis buffer and analyzed within 8 h. HbA_{1c} was measured on the COBAS MIRA Plus autoanalyzer (Roche Diagnostic Systems, Basel, Switzerland), as described by manufacturer.

2.6. Pancreas histology

After euthanasia with isoflurane at the end of the study period, the pancreas was removed, weighed and cut into 20–30 pieces with a pair of scissors. The pieces were quickly lined up according to the principle of the smooth fractionator with the biggest piece in the middle and decreasing sized pieces to each side [41]. Every third piece was collected and randomly two of the collections were assigned for making paraffin blocks and the last collection was quickly frozen on dry ice for doing insulin extraction.

2.7. Immunohistochemistry

The tissue for embedding in paraffin was fixed in 4% paraformaldehyde overnight. The tissue was then transferred to 70% ethanol, embedded in paraffin, and sectioned at 3–4 µm using the 2055 Autocut microtome from Leica Instruments GmbH (Wetzlar, Germany). In brief, the sections were deparaffinized in xylene and rehydrated, and immunostained using the following protocol for ki67 and insulin: microwave oven treatment at 650 W for 3 min and 160 W for 15 min in Tris–EGTA buffer, pH 9, followed by cooling for 20 min. The sections were then transferred to TBS for five min, blocked in 0.5% hydrogen peroxide for 20 min, blocked with avidin followed by biotin (X0590, DAKO, Glostrup, Denmark) for 10 min each, and blocking in 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Chester, PA) for 30 min. Next, the slides were incubated overnight