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Long-term liraglutide treatment is associated with increased insulin content and secretion in β-cells, and a loss of α-cells in ZDF rats☆

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ABSTRACT

The ultimate treatment goal of diabetes is to preserve and restore islet cell function. Treatment of certain diabetic animal models with incretins has been reported to preserve and possibly enhance islet function and promote islet cell growth. The studies reported here detail islet cell anatomy in animals chronically treated with the incretin analog, liraglutide. Our aim was to quantitatively and qualitatively analyze islet cells from diabetic animals treated with vehicle (control) or liraglutide to determine whether normal islet cell anatomy is maintained or enhanced with pharmaceutical treatment. We harvested pancreata from liraglutide and vehicle-treated Zucker Diabetic Fatty (ZDF) rats to examine islet structure and function and obtain isolated islets. Twelve-week-old male rats were assigned to 3 groups: (1) liraglutide-treated diabetic, (2) vehicle-treated diabetic, and (3) lean non-diabetic. Liraglutide was given SC twice daily for 9 weeks. As expected, liraglutide treatment reduced body weight by 15% compared to the vehicle-treated animals, eventually to levels that were not different from lean controls. At the termination of the study, blood glucose was significantly less in the liraglutide-treated rats compared to vehicle treated controls (485.8 ± 22.5 and 547.2 ± 33.1 mg/dl, respectively). Insulin content/islet (measured by immunohistochemistry) was 34.2 ± 0.7 pixel units in vehicle-treated rats, and 54.9 ± 0.6 in the liraglutide-treated animals. Glucose-stimulated insulin secretion from isolated islets (measured as the stimulation index) was maintained in the liraglutide-treated rats, but not in the vehicle-treated. However, liraglutide did not preserve normal islet architecture. There was a decrease in the glucagon-positive area/islet and in the α-cell numbers/area with liraglutide treatment (6.5 cells/field), compared to vehicle (17.9 cells/field). There was an increase in β-cell numbers, the β- to α-cell ratio that was statistically higher in the liraglutide-treated rats (24.3 ± 4.4) compared to vehicle (9.1 ± 2.8). Disrupted mitochondria were more commonly observed in the α-cells (51.9 ± 10.3% of cells) than in the β-cells (27.2 ± 4.4%) in the liraglutide-treated group. While liraglutide enhanced or maintained growth and function of certain islet cells, the overall ratio of α- to β-cells was decreased and there was an absolute reduction in islet α-cell content. There was selective disruption of intracellular α-cell organelles, representing an uncoupling of the bihormonal islet signaling that is required for normal metabolic regulation. The relevance of the findings to long-term liraglutide treatment in people with diabetes is unknown and should be investigated in appropriately designed clinical studies.

1. Introduction

The incretin hormones are peptides released by the gastrointestinal tract in response to the ingestion of food. These hormones enhance insulin secretion and regulate glucose homeostasis by inhibiting glucagon secretion, slowing gastric emptying, and controlling satiation, ultimately controlling body weight [1]. GLP-1 is one of the major incretin hormones that rapidly stimulates insulin release. Several peptide drugs have been designed to mimic the action of GLP-1, among them exenatide and liraglutide, with the long-term goal of improving blood glucose regulation and enhancing weight loss. In 2010, the Food and Drug Administration

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; EM, electron microscope; ER, endoplasmic reticulum; GLP-1, glucagon-like peptide-1; HbA1c, glycated hemoglobin; HRP, horseradish peroxidase; IACUCI, nstitutional Animal Care and Use Committee; IF, immunofluorescence; IHC, immunohistochemistry; IP, intraperitoneal injection; LSD, least significant difference; NDS, normal donkey serum; PBS, phosphate buffered solution; ZDF, Zucker diabetic fatty.

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approved liraglutide for daily, chronic treatment of adults with type 2 diabetes [2]. Liraglutide has 97% homology in amino acid sequence with endogenous GLP-1 and is resistant to enzyme inactivation [3]. Administration of liraglutide to people with type 2 diabetes significantly reduced fasting glucose levels, decreased HbA1c values, and decreased body weight [4]. There is indirect evidence, based on the long-term stability of HbA1c levels, that treatment with GLP-1 stabilizes islet cell function in humans, and may slow the usual progression of insulin depletion over time in type 2 diabetes [5].

Diabetic ZDF rats, and those with 60% pancreatectomies, treated with liraglutide have increased β-cell mass, which is more prominent in the most obese animals as compared to the glucose-controlled animals [6]. β-Cell glucose sensitivity was restored with GLP-1 treatment, resulting in increased insulin secretion in rats. Conversely glucagon secretion was inhibited [7,8]. The glucagon inhibition is thought to be due to the immediate effect of the incretin, as direct exposure of an α-cell culture line to GLP-1 also inhibited glucagon release [9]. In conditions where glucagon production and secretion are excessive, such inhibition may be beneficial [10]. While improved α-cell regulation may be a goal, abnormal loss of α-cells and/or their function would not be optimal for glucose homeostasis.

The increase in islet cell mass with improvement in glycemic control raises the possibility that treatment with GLP-1 analogs anatomically and, to some extent, physiologically reverses the insulin deficiency characteristic of type 2 diabetes. The purpose of this study was to determine whether the islets remaining after long-term GLP-1 administration possessed normal morphology and function using an animal model of type 2 diabetes with emphasis on α-cells. Arguably, any treatment that enhances islet cell function in diabetes should preserve or enhance normal islet cell structure and function.

2. Methods

2.1. Animal model

Male ZDF rats of 12 weeks of age on arrival were assigned to 3 groups: (1) liraglutide-treated diabetic (strain code #370, obese fa/fa), (2) vehicle-treated diabetic (strain code #370, obese fa/fa), (3) lean non-diabetic (strain code #380, lean fa/+). Rats were purchased from Charles River Laboratory (Saint Louis, MO) and allowed one week for acclimation prior to experimentation. The rats received husbandry services at the Laboratory Animal Resources facility at the University of Kansas Medical Center. The rats were allowed food (Purina 5008) and water *ad libitum* and were maintained on a 12:12 light–dark cycle. All animal procedures were performed according to the IACUC guidelines of the University of Kansas Medical Center and the studies were approved by the local animal use committee.

Body weights were recorded weekly on all rats. Blood glucose levels were assessed by a digital blood glucose meter (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN). Weekly blood glucose levels were calculated as an average of daily levels measured for a given week. Hemoglobin A1c (HbA1c) levels were determined by a home evaluation unit (A1C Now At Home System, Bayers, Pittsburgh, PA). When rats had blood glucose or HbA1c levels higher than the range of detection by the instrument, statistical analysis of the data followed previously published procedures [11,12]. Briefly, the highest detectable value for blood glucose (600 mg/dl) was substituted for the missing data.

Animals were administered subcutaneous liraglutide for 9 weeks bid (6 mg/ml; dissolved in vehicle of disodium hydrogen phosphate dihydrate, polyethylene glycol and H_2O) at a final dose of 0.225 μg/g body weight. At the termination of the study, rats were over-anesthetized with tribromoethanol and the pancreas or isolated islets processed according to the methods listed below.

2.2. Islet isolation

At the termination of the study the pancreata were cannulated *in situ via* the common bile duct, and distended with cold collagenase (CLS-1, Worthington Biochemical in Leibovitz L15 at 450 U/ml). The distended pancreas was excised and incubated for 20–30 min with gentle tumbling in a 37 °C incubator. The contents of the tube were placed in diluted ice-cold Hank's Balanced Salt Solution (HBSS) containing 5% fetal calf serum. The digest was allowed to settle at $1 \times g$ and the supernatant removed and the process repeated. The washed digest was passed through a 500 μm screen and sedimented for 1 min at $300 \times g$. The pellet was mixed with 10 ml of 1.110 g/ml Histopaque (density = 1.1085, Sigma Diagnostics Inc.) and centrifuged. The islets floating on the interphase of the gradient were collected, sedimented, and cleaned using a sterile 40 μm mesh cell strainer and HBSS with 5% fetal calf serum. Islets were placed into CMRL 1066 containing 2 mM glutamine, 10% fetal bovine serum and 1% antibiotic/antimycotic solution.

2.3. Insulin secretion

Islets were placed in duplicate wells and assigned to three groups: basal glucose (1 mM), low glucose (3 mM), and high glucose (20 mM). All wells were preincubated for 30 min in RPMI 1640 containing 3 mM glucose in a 37 °C containing 5% CO_2. After preincubation, media were removed from each well and discarded. Basal, low or high glucose solutions were added to the duplicates. After 30 min static incubation in the 37 °C and 5% CO_2, the islets were sedimented and the conditioned medium was collected to determine insulin content and frozen at −80 °C. Insulin concentration was determined by ELISA (ALPCO, Windham, NH). The stimulation index was calculated as the amount of insulin released at low or high glucose divided by the basal insulin amount.

2.4. Electron microscopy

Electron microscopy was conducted using 2 mm sections of pancreatic tissue fixed in 2% glutaraldehyde. Samples were rinsed twice in 0.1 M sodium cacodylate buffer for 10 min prior to post fixation in 1% osmium tetroxide for 1 h. Rinsing with distilled water was followed by a gradual ethanol dehydration (30%, 70%, 80%, 95%, 100%) for 10 min each. Samples were rinsed twice in propylene oxide for 15 min prior to being infiltrated in a mixture of propylene oxide and Embed 812 resin (Electron Microscopy Sciences, Ft. Washington, PA) overnight. BEEM capsules were used to embed the samples in fresh resin prior to curing overnight in a 70 °C oven. Thin sections, 80 nm in diameter, were cut using a Leica UCT ultramicrotome and placed on 300 mesh thin bar grids. Contrast was applied to the sections by adding uranyl acetate followed by Sato's lead stain. Images of ZDF pancreatic islets were captured from random tissue sections using a J.E.O.L JEM 1400 transmission electron microscope.

Analysis of the EM images was completed by a blinded investigator who had not participated in the animal portion of the study. Analysis included calculating the number of insulin granules, and evaluating the mitochondria quality. Images were analyzed in Adobe Photoshop (Adobe Systems, Inc.) or Scion Image (Scion Corp.). Insulin granules were counted manually within an assigned area on each micrograph using previously published procedures [13,14]. The insulin granules were easily identified by their halo presentation, making them unique from the other secretory granules such as those containing glucagon or somatostatin. The level