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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

of intact mitochondria was determined using the published Mitochondrial Quality Index [15]. Briefly, individual mitochondria were assigned one of the following grades: 1 – fully intact, 2 – disruption of the cristae (typically from swelling or loss of integrity of the inner membrane), 3 – disruption of the outer mitochondrial membrane, and 4 – disruption of the cristae and the outer membrane.

2.5. Immunohistochemistry and immunohistofluorescence

The pancreata were rapidly harvested and fixed in 10% normal buffered formalin using our published protocols [16]. Sections were blocked in 10% normal donkey serum (NDS), 1.0% bovine serum albumin (BSA), and 0.03% Triton X-100 diluted in 0.1 M PBS, pH 7.4 for 30 min. Both primary and secondary antibodies were diluted in 1% NDS, 1% BSA, and 0.03% Triton X-100. Slides were mounted with anti-fading agents Gel/Mount (Biomeda, Foster City, CA, PA, #M01). The primary antibodies consisted of: anti-insulin (1:100, Abcam, Cambridge, MA, #ab7842), anti-glucagon (1:300, Abcam, #ab10988), anti-somatostatin (1:300, Abcam, #ab53165) and anti-Ki67 [SP6] proliferation marker (1:100, Abcam, #ab16667), anti-proinsulin (1:200, Abcam, #ab50805) and CD34 (1:200, Abcam, #ab812894). Corresponding secondary antibodies were conjugated with Dylight 488 (1:400, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, #706-485-148), Alexa 555 (1:400, Molecular Probes, Eugene, OR, #A31570), and Alexa 647 (1:400, Molecular Probes, #A31573). Images were collected using a Nikon C1Si confocal microscope.

Insulin immunohistochemistry (IHC) was completed on paraffin embedded pancreatic tissue using anti-insulin (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, #sc-9168) and Histostain Plus Rabbit Primary (Invitrogen, Frederick, MD, #85-6143) in combination with AEC (3-amino-9-ethyl-carbazole) chromogen as a substrate of Horseradish Peroxidase (HRP) enzyme. Slides for IHC were prepared in the same manner as for IF. After the permeabilization step, a blocking solution was applied. The IHC procedure was conducted according to the manufacturer instructions.

After staining, the slides were placed on coverslips in mounting medium Clear-Mount with Tris Buffer (Electron Microscopy Sciences, Hatfield, PA, #17985-12). The specificity of insulin immunoreactivity was confirmed by omitting the primary antibodies from some of the collected sections. The staining was observed by light microscopy (Nikon Eclipse 80i). Images were analyzed with Ps Adobe Photoshop CZ4 extended software, by determining the average pixel value of staining per cell or per islet. Background staining was subtracted from each value.

Islet density was defined as the number of islets per field using a 20× objective. Islet size was estimated by measuring the diameter of the islet under the software Nikon EZ-C1 3.0 FreeViewer. For the cell composition analysis, the relative proportion of α-, β- or δ-cells in one islet was evaluated by counting the number of individual types of cell and dividing by the total sum of endocrine (α, β and δ) cells per islet. Gross islet morphology was categorized as either (1) intact, (2) disrupted or (3) scattered cells. Intact islets were defined as having a distinct border with a circular or ellipsoidal shape. Disrupted islets also contained an identifiable border, but were missing sections of cells stained for either insulin, glucagon, or somatostatin. For an islet to be classified as disrupted, at least 25% of the area had to lack staining for insulin, glucagon or somatostatin. Scattered cells were defined as fewer than 10 insulin, glucagon, or somatostatin positive cells in an area. Multiple sequential sections were analyzed to make sure that a scattered appearance was not the tip of an islet, but truly a small number of endocrine cells alone. All islet morphology, density, and pixel values were analyzed by a blinded investigator who did not participate in the *in vivo* portion of the study or in the collection of the images.

2.6. Statistics

A *t*-test was used to compare total insulin content. Body weight and blood glucose data were analyzed using repeated measures ANOVA. Insulin secretion was analyzed using one-way ANOVA. For the immunostaining experiments, nested ANOVA was utilized. All figures include means ± SE. *p*-Value, defined as <0.05, was considered statistically significant.

3. Results

3.1. Metabolic effects

Over the 9 week course of the study, the body weight of the diabetic ZDF rats increased over the matched lean animals (Fig. 1A, $p < 0.03$). Administration of liraglutide was associated with a body weight that was not statistically different from the lean animals, and was less than the vehicle-treated diabetic rats. Blood glucose levels increased in both diabetic groups compared to the lean non-diabetic controls at each time point tested (Fig. 1B; $p < 0.001$). Liraglutide significantly reduced the blood glucose levels in the treated ZDF rats as compared to the vehicle-treated animals.

At the termination of the study, glucose tolerance tests were administered to the liraglutide and vehicle-treated animals. There was no difference in the fasting glucose measurements prior to the initiation of the experiment (Fig. 1C). However, the blood glucose of the vehicle-treated animals rose to levels above the detection limits of the procedure after the glucose injection (at 30 and 60 min). During the same time points, the liraglutide-treated animals had mean blood glucose values of 529 ± 50 and 542 ± 51 mg/dl, respectively. There was no difference between groups in the glucose values 2 h after the injection of glucose.

3.2. Insulin content

Immunohistochemistry was used to identify islets from the pancreas of each group of rats. Obviously, it was difficult to find intact islets from the 2 diabetic groups. The results demonstrated differences in the insulin staining intensity between the 3 groups. As expected, the non-diabetic lean controls had the most intense insulin staining (Fig. 2A), followed by the liraglutide-treated animals (Fig. 2C). Pancreatic sections from the vehicle-treated animals had the least insulin staining (Fig. 2B). The color intensity was calculated for individual β-cells within the islets and the means are provided in Fig. 2D. Analysis of proinsulin staining from immunofluorescence images revealed the highest levels of proinsulin in the liraglutide-treated rats (pixel intensity units 44.3 ± 3.7) compared to 37.3 ± 2.1 for the diabetic vehicle-treated rats, and 29.2 ± 3.0 in the lean controls ($p < 0.005$).

Consistent with the immunohistochemistry data showing greater insulin staining, the density of insulin granules in the β-cells of islets from the liraglutide-treated animals increased as compared to the controls. Insulin granules were identified by their hallmark halo appearance in electron micrographs, illustrated by arrows in Fig. 3A. The β-cells from the liraglutide-treated animals possessed a higher density of insulin granules per β-cell area than the vehicle-treated animals (Fig. 3B).

Insulin secretion was measured from isolated islets from the 3 groups in conditions of 1 mM, 3 mM and 20 mM glucose. Islets from the lean animals showed a typical response with little stimulation at 3 mM glucose, but a significant increase in insulin release at 20 mM (Fig. 3C, $p < 0.01$). There was no significant increase in the amount of insulin released from the islets of vehicle-injected rats in response to 20 mM glucose. The liraglutide-treated rats had islets with a stimulation index that was not statistically different from

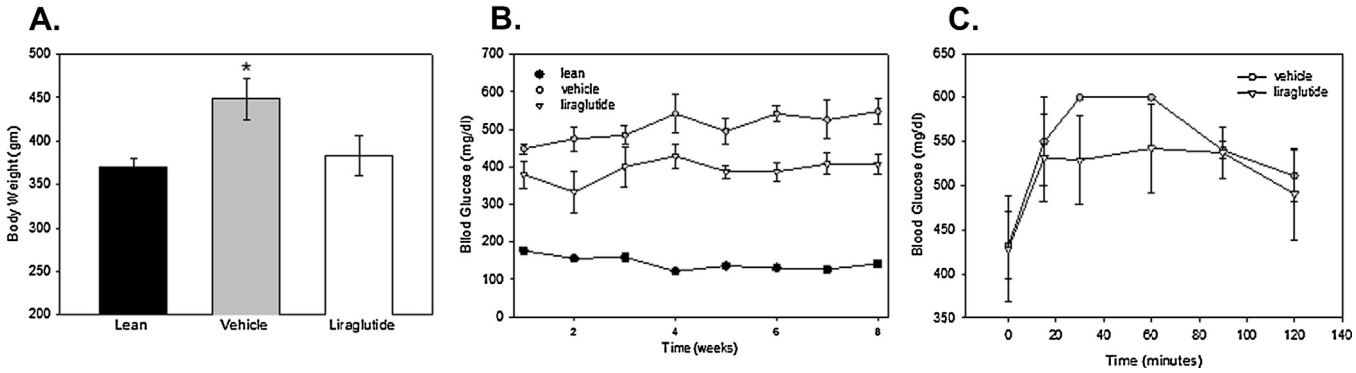

Fig. 1. Metabolic characteristics. (A) Body weights for the vehicle-treated animals were higher than the lean non-diabetic controls or the liraglutide-treated diabetic rats ($p < 0.05$). (B) The non-fasting blood glucose levels for the 2 diabetic groups were significantly higher than the lean controls at each time point measured ($p < 0.001$). The blood glucose levels were lower in the liraglutide-treated group compared to the vehicle-treated group ($p < 0.02$). (C) Glucose tolerance tests illustrated the rapid increase in blood glucose levels after an IP bolus injection of glucose. In fact, the glucose meter reached its maximum detectible level within 15 min of the injection. None of the liraglutide-treated animals reached the maximum levels.

the lean control rats, but was higher than the 3 mM exposure in the islets from liraglutide-treated rats ($p < 0.001$).

3.3. Islet morphology

Morphological differences between groups clarify the cellular changes that occur with diabetes. The lean controls had healthy appearing β-cells with normal islet morphology consisting of centrally located β-cells and α- and δ-cells on the periphery (Fig. 4A). This cellular architecture was consistent with 88% of all islets from the lean controls demonstrating a β-cell core pattern ($n = 41$ islets from 3 rats). Only 6% of the islets from the diabetic vehicle-treated rats were organized with the β-cell core ($n = 113$ islets from 4 rats). Liraglutide did not improve the cellular architecture, because only 1% of the islets from the treated group had β-cell cores ($n = 71$ islets from 4 rats).

In addition to the location of specific endocrine cell types within the islets, the overall shape of the islets was altered with diabetes. In the diabetic animals, the islets often had a disrupted appearance with large areas in the islet core void of endocrine cells or areas of scattered endocrine cells amongst exocrine tissue (Fig. 4B and C). We identified 3 major islet morphology categories: (1) intact, (2) disrupted and (3) scattered cells. In the lean animals, the majority of the islets were intact with approximately 15% classified as disrupted islets or scattered endocrine cells (Table 1). A majority of the islets in the vehicle-treated rats were disrupted, with entire sections of islets missing endocrine cells as shown in Fig. 4B. The liraglutide-treated animals had a majority of islets classified as

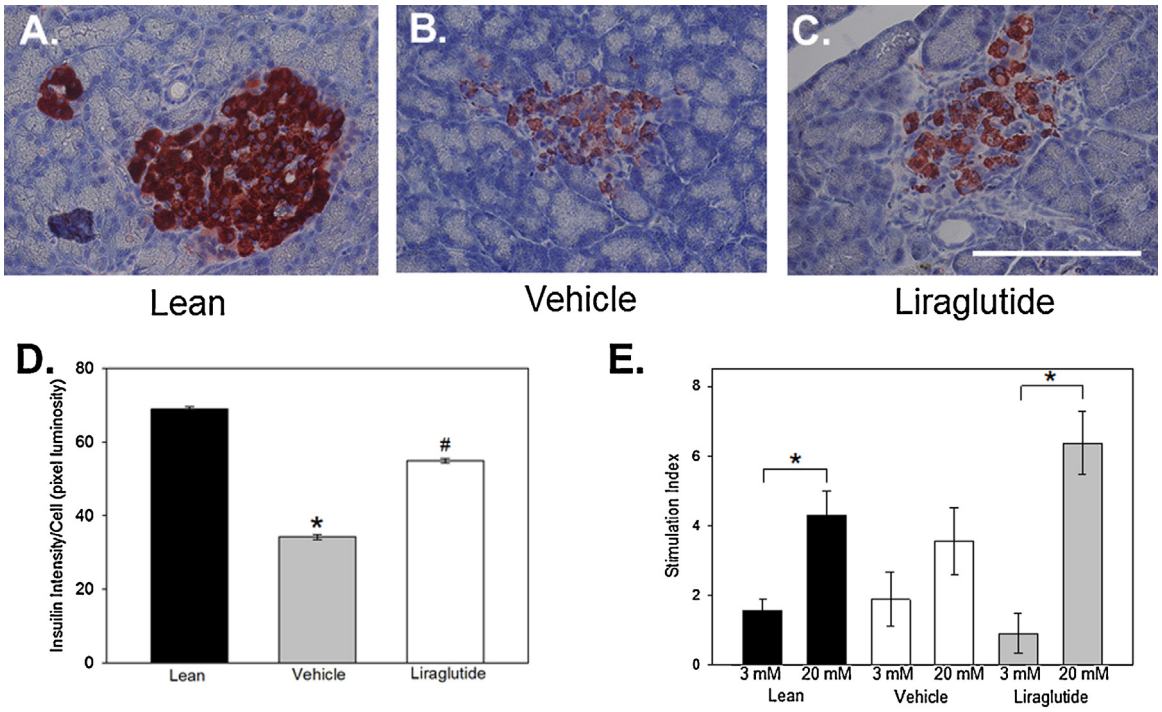

Fig. 2. Cellular insulin levels. (A) Immunohistochemistry of pancreatic sections stained for insulin shows the most intense insulin staining in an islet from a lean non-diabetic rat. (B) Islets from the vehicle-treated animals possessed weak insulin staining. (C) The islets from the liraglutide-treated animals illustrated more intense immunostaining than the vehicle group. (D) The graph summarizes these findings, showing the insulin intensity/cell with the liraglutide group possessing more insulin/cell than the vehicle-treated animals, but statistically less than the non-diabetic lean controls. The graph summarizes analysis of an average of 22 islets/group from 9 sections. *Difference between the lean controls and the vehicle-treated group ($p < 0.001$). #Difference between the vehicle- and liraglutide-treated groups ($p < 0.05$). Scale bar = 100 μm for all images.

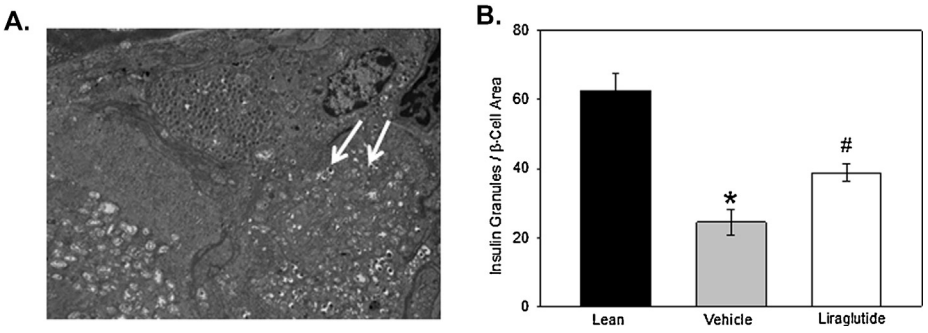

Fig. 3. Insulin granules. (A) Micrograph of sample islet cells from a vehicle-treated animal. White arrows indicate insulin granules (magnification = 1500×). (B) Analysis of insulin granule density demonstrated that the 2 diabetic groups had a lower granule density than the lean control animals. An average of 36 islets/group were analyzed from 3 rats/group (*$p < 0.001$ and #$p < 0.05$). (C) The glucose-stimulated insulin release (stimulation index) was measured from isolated islets. The graph summarizes the mean of 2 independent trials (2–3 replicates). Islets from the lean control animals and the liraglutide-treated group had a significant increase in insulin release in response to 20 mM glucose compared to 3 mM glucose (*$p < 0.01$).

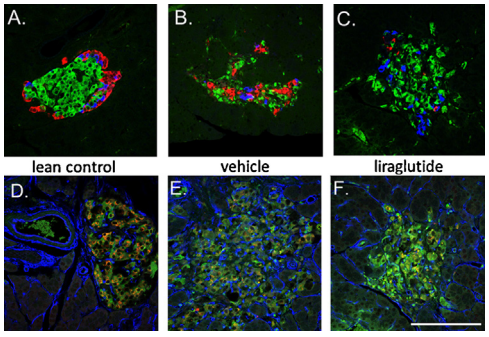

Fig. 4. Morphology of islets. (A) Antibody-labeled islets from non-diabetic lean control animals illustrated a normal morphology. (B) The islets from the vehicle-treated diabetic rats had large areas of missing cells, indicating disrupted islets. (C) The pancreatic sections from liraglutide-treated animals appeared similar to the vehicle-treated group. Counter staining with DC34 for blood vessels (shown surrounding the islet) and insulin and proinsulin (staining the islets) from lean controls (D), vehicle-treated (E) and liraglutide-treat (F) animals all had similar vascularity with less proinsuilin in the 2 diabetic groups (vehicle and liraglutide-treated). Scale bar = 100 μm for all images.

either disrupted or scattered cells (Table 1). There was no statistical difference between the 2 diabetic groups (vehicle or liraglutide-treated) for any of the classifications compared. However, both groups were statistically different from the islets obtained from the non-diabetic lean animals, except for the classification of scattered endocrine cells.

The disrupted appearance of many of the islets from the 2 diabetic groups led to questions about the vascularity of the islets associated with diabetes and liraglutide treatment. Pancreatic sections were stained with CD34 to identify vessels surrounding and penetrating the islets. Fig. 4D shows the vessel staining around an islet from a lean control rat. The green cells within the islets are positive for insulin (β-cells) with proinsulin additionally stained in red/orange. As has been shown in the past, blood vessels appear to align with the insulin-containing portion of each β-cell and opposite the proinsulin location in the healthy rats [17]. The vehicle-treated rats had evidence of abundant blood vessels that penetrated throughout the islets with a more scattered distribution of proinsulin within each cell (Fig. 4E). Liraglutide did not change the appearance or density of blood vessels within the islets (Fig. 4F). More proinsulin was located in β-cells near blood vessels in the liraglutide-treated animals than in the vehicle treated diabetics.

Table 1
Islet morphological categories.

	Intact	Disrupted	Scattered
Lean	84.1 ± 5.3*	7.9 ± 4.1*	7.7 ± 3.3
Diabetic vehicle	21.8 ± 12.0	67.33 ± 15.3	11.2 ± 5.0
Diabetic liraglutide	12.4 ± 5.5	47.0 ± 7.1	40.4 ± 8.2*

The percentage of intact islets was greatest in the lean, non-diabetic group. Islets from the two diabetic groups showed substantial numbers of disrupted islets, in which the outer capsule of the islet was damaged and lacked a smooth border. The percentage of islets with disrupted outer rims was the same as small clusters of scattered endocrine cells in the pancreata from liraglutide-treated animals. n = slides analyzed (lean = 8, vehicle = 8, liragluide-treated = 7).

* $p < 0.05$.

3.4. α-Cells and glucagon content

There was no significant difference in the percentage of β-cells present in individual islets between groups (68.1 ± 2.5% in lean controls, 72.8 ± 2.0% in vehicle-treated, and 67.4 ± 3.2% in liraglutide-treated rats). However, when the total area per microscopic field stained for glucagon was calculated, there was far less glucagon in the liraglutide-treated animals than in either of the other two groups (Fig. 5A). A difference in glucagon staining per islet could be due to changes in cell size (hypertrophy or atrophy) or in cell number. To clarify, individual α- and δ-cell numbers were counted using 40× images and the liraglutide group again had significantly fewer α-cells/field than the other two groups (Fig. 5B). There was no difference in the number of δ-cells/field (lean = 17.4 ± 5.6; vehicle = 14.2 ± 2.3; liraglutide = 12.3 ± 1.6). The loss of α-cells resulted in an increase in the β-cell/α-cell ratio (Fig. 5C). In addition, the intensity of the glucagon staining within each α-cell was significantly lower in the two diabetic groups compared to the lean controls (Fig. 5D). In δ-cells, there was significantly less somatostatin staining in the liraglutide treated cells compared to the other two groups (Fig. 5E).

3.5. Cell proliferation

To begin to identify the mechanisms responsible for the changes in α- to β-cell ratios, we identified proliferating cells using Ki67, a well-characterized nuclear protein essential for proliferation. In the lean, non-diabetic animals positive Ki67 staining was present in 14% of all of the α-cells (Table 2). In contrast, only half that many α-cells were proliferating in the vehicle-injected diabetic rats, and there were no proliferating α-cells in the liraglutide-treated animals. In contrast, the percentage of β-cells actively proliferating was higher in both diabetic groups compared to the non-diabetic group. The liraglutide group had lower β-cell proliferation rates than the vehicle-treated rats (Table 2).

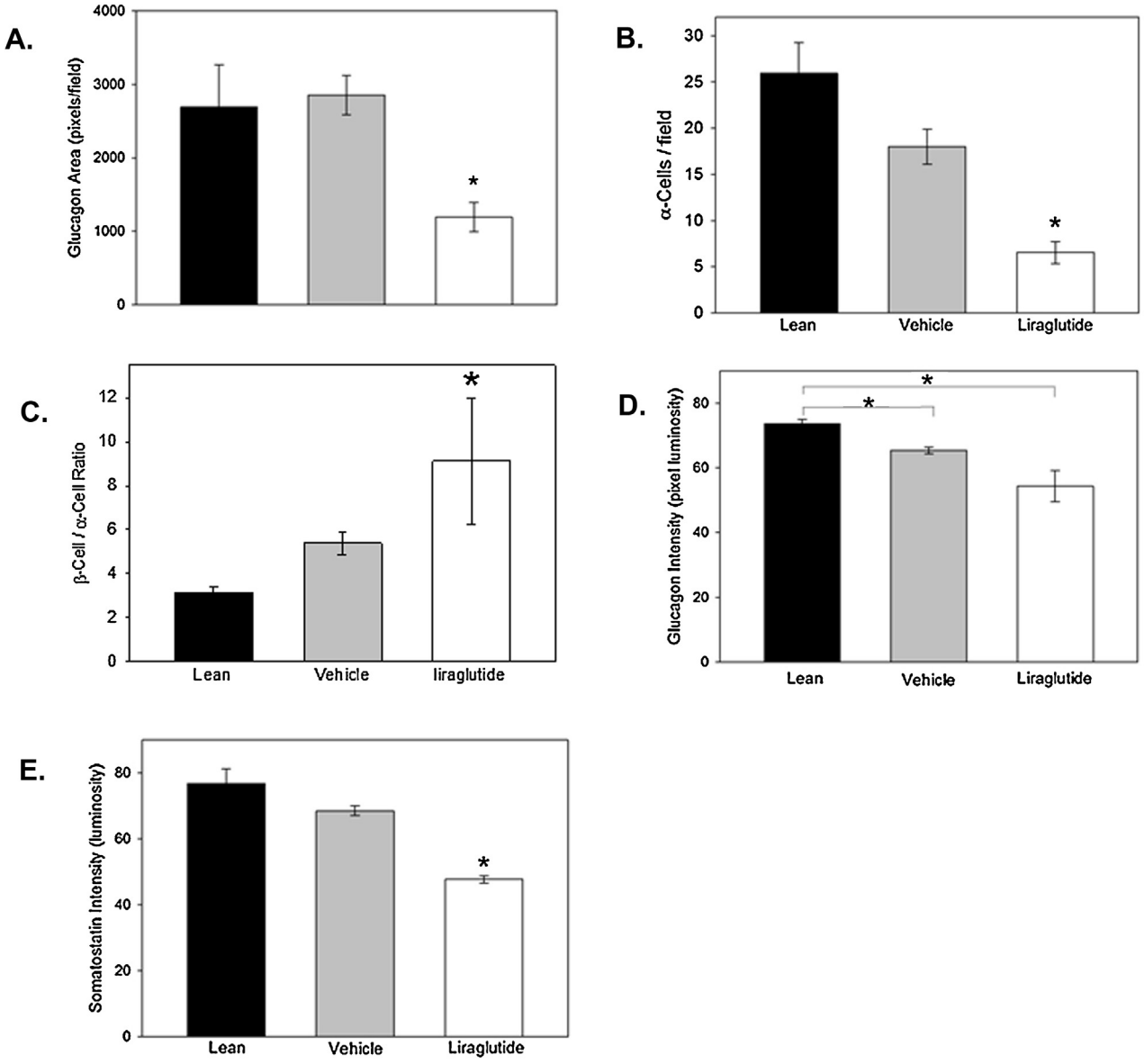

Fig. 5. α-Cell survival and glucagon content. (A) Analysis of triple stained images confirmed that there was less glucagon staining when measured as total pixels. An average of 7 sections/group were analyzed with 16.2 islets/section (*$p < 0.001$). (B) There were fewer glucagon-positive α-cells per field. An average of 44 fields from 9 sections/group were analyzed (*$p < 0.01$). (C) The β-cell to α-cell ratio was highest in the liraglutide-treated animals. An average of 7 sections/group were analyzed with 16.2 islets/section (*$p < 0.05$). (D) The intensity of the glucagon staining within each α-cell was decreased in the liraglutide-treated animals. An average of 5 sections/group were analyzed with 35 cells/islet (*$p < 0.01$). (E) The intensity of the somatostatin immunostaining within each δ-cell was significantly decreased in the liraglutide-treated animals. An average of 8 sections/group were analyzed from 12 cells/islet (*$p < 0.001$).

Table 2
β- and α-cell proliferation rates.

	α-Cells	β-Cells	Other
Lean	14.3 ± 1.1	28.6 ± 5.9	64.3 ± 8.5
Diabetic vehicle	6.7 ± 0.9	80.0 ± 6.5*	82.2 ± 1.0*
Diabetic liraglutide	0.0 ± 0.0	56.6 ± 2.5	56.8 ± 4.7

Positive Ki-67 staining indicated proliferating cells. In the lean, non-diabetic animals there was a low level of proliferating α- or β-cells compared to other cells in the islet. However, in the diabetic, vehicle-administered animals the β-cells had a high level of proliferation. Administration of liraglutide significantly reduced the proliferating β-cells with no proliferation found in α-cells. $n = 17$ islets from lean rats, 15 diabetic vehicle rats, and 17 diabetic liraglutide rats.

* $p < 0.05$ for comparison within β-cells and other cell types.

3.6. Mitochondrial ultrastructure

Given the loss of α-cells in the islets of the liraglutide-treated rats, we investigated the ultrastructure of these cells. The quality of the mitochondria, based on EM micrographs, was scored using the Mitochondrial Quality Index [14]. The sample image of an α-cell from a lean control rat illustrates healthy mitochondria given a score of 1 (Fig. 6A). α-Cells from vehicle- and liraglutide-treated animals illustrate mitochondria with scores of 2, 3 and 4 (Fig. 6B and C). In β-cells, only the cells from lean control animals had a predominance of healthy mitochondria with no signs of completely disrupted mitochondria (Fig. 6D). Cells from the vehicle and liraglutide treated groups had nearly equal numbers of mitochondria in all 4 quality categories. Likewise, in α-cells from the lean control animals, the majority of mitochondria were completely intact (Fig. 6E). In contrast, α-cells from the liraglutide-treated animals had a much

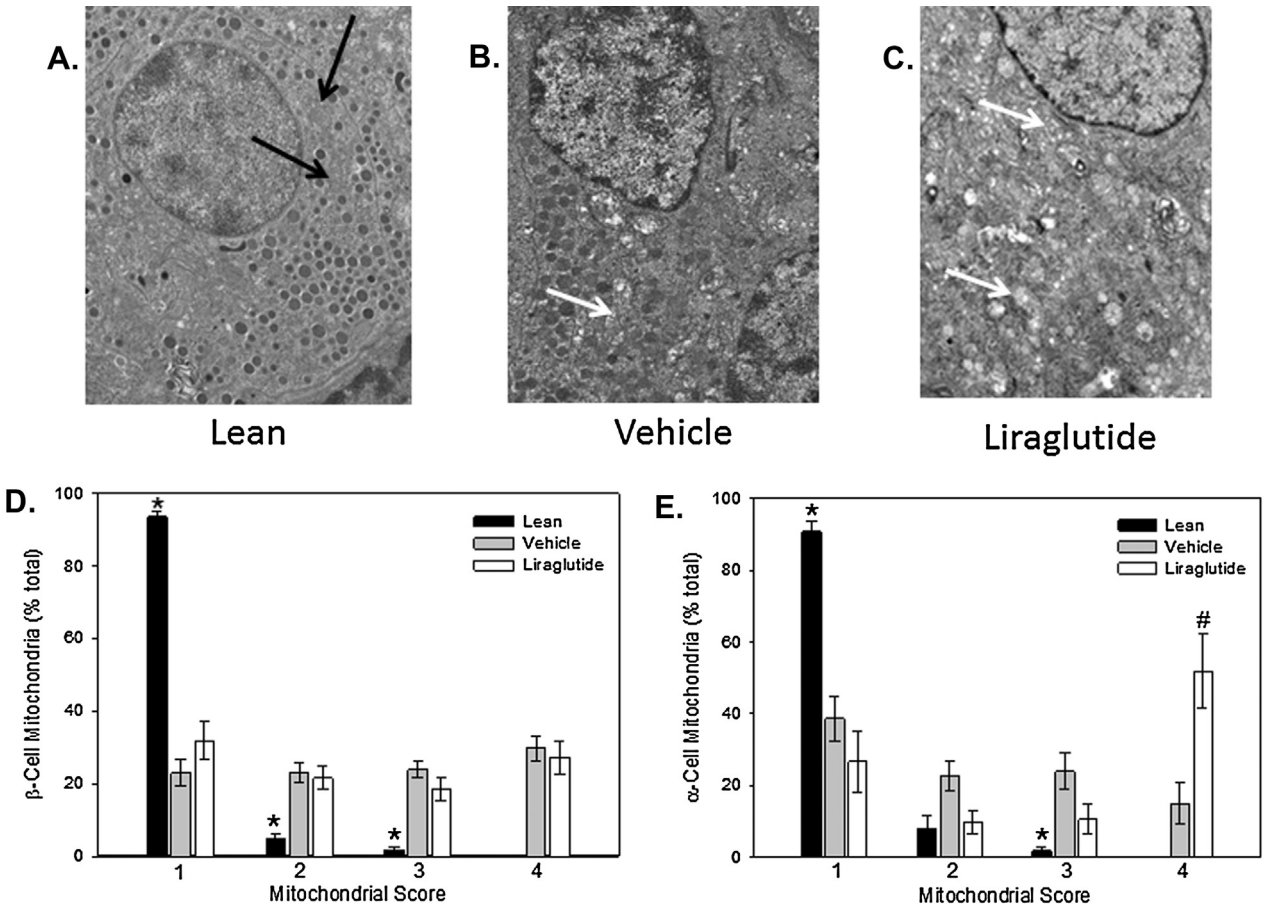

Fig. 6. Mitochondria quality index score. (A) Electron micrographs illustrated the intact mitochondria in an α-cell from a lean control animals (black arrows). (B and C) Vehicle and liraglutide-treated animals had α-cells with disrupted mitochondria with MQI scores of 2–4 (white arrows). (D) There was no difference in the MQI score for β-cells between the vehicle and liraglutide-treated groups. Mitochondrial integrity from the lean controls was significantly higher compared to the other 2 groups ($p < 0.001$). (E) α-Cell mitochondrial integrity was also greatest in the lean control animals ($^*p < 0.001$). There were significantly more disrupted mitochondria (score of 4) in the liraglutide-treated animals compared to the vehicle-treated group ($\#p < 0.001$). All images analyzed were collected at 1500× magnification.

higher percentage of the mitochondria that were completely disrupted (score of 4) compared to the vehicle-treated rats (Fig. 6E).

4. Discussion

The results of this study indicate that long-term liraglutide treatment reduced weight gain and lowered blood glucose in severely diabetic ZDF rats. However, an excessive loss of α-cells, without a loss in δ-cell number, was observed with markedly abnormal islet morphology. In spite of the bihormonal hypothesis first proposed by Unger and Orci in 1975 [18], little has been done to explore the role of the α-cell and glucagon secretion with GLP-1 analogs. It has been known for over 15 years that GLP-1 suppresses glucagon release [19], and this suppression is not dependent on the increased insulin secretion stimulated by the compound, as it occurs even in cultured α-cell lines [9]. Furthermore, the increase in islet size reported in some studies with GLP-1 treatment was due to the stimulation of β-cell proliferation [20], with no observable changes in other cell types. Liraglutide was shown to increase β-cell mass by increasing the cellular kinetics and suppressing ER stress in db/db mice after only 2 weeks of treatment. Our study, conducted for 9 weeks on severely diabetic rats, showed islets with abnormal morphology in both the vehicle and liraglutide-treated groups with excessive loss of α-cells in the treated animals.

In ZDF rats, liraglutide has been reported to initially reduce body weight, but the animals gained the weight back, so that within 6 weeks of treatment they were statistically heavier than the vehicle-treated rats [6]. In our study, 9 weeks of treatment resulted in a significant decline in weight compared to animals exposed to vehicle alone, resulting in body weights that were not statistically different from the lean controls. Similar consistent losses in body weight have been identified in db/db mice [21]. In agreement with our findings, previous studies using ZDF rats and liraglutide monotherapy noted a decline in blood glucose levels with liraglutide, but this reduction did not bring the glucose levels down to nondiabetic values [6,22].

The endocrine function of the islet is a delicate balance of both paracrine, endocrine, neural and possibly vascular communication. Thus, changes in the cellular equilibrium must be investigated since they may ultimately affect the clinical response to chronic exposure of high levels of GLP-1. In another study employing long-term liraglutide treatment, there was no increase in the β-cell mass or Ki-67 staining in male ZDF rats [22]. In Sturis et al., proliferating β-cells numbers actually dropped with 2 weeks of liraglutide administration, but then returned to vehicle levels by week 6 [6]. We show a decline in β- and α-cell proliferation along with a decline in all cell proliferation in the islets with liraglutide treatment compared to the vehicle group, in sharp contrast to the current dogma concerning β-cell proliferation and GLP-1 analogs. It is important to note that the greatest reported increase in β-cell proliferation with liraglutide has been in primary monolayer cultures from newborn rats [20], and not *in vivo* studies. Even when there was a

reported increase in proliferating β-cells in intact islets in response to liraglutide, those proliferating cells were still extremely rare events in both human and rat islets [23,24]. Thus, the exact level of β-cell proliferation in humans in response to liraglutide is still unclear.

The impact of GLP-1 and its analogs on α-cells has been uncertain. In isolated rat and human islets, GLP-1 exposure significantly decreased the content and release of glucagon from α-cells [8,25]. While in the perfused rat pancreas, GLP-1 had no effect on glucagon release [26]. In humans, liraglutide caused significantly lower glucagon levels when fasting and following meals [27] especially protein-rich meals [28]. In one of the most interesting experiments, humans were subjected to a step-wise hypoglycemic clamp and plasma glucagon levels sampled. At both 4 and 5 mM glucose, the subjects given liraglutide showed significantly less glucagon secretion than the placebo group [29,30]. At even lower glucose levels (down to 2.7 mM) there was either no effect of liraglutide, or the liraglutide group actually secreted more glucagon than the placebo group [30]. However, it is important to note that those tests utilized acute (30 min) exposures to GLP-1 or GLP-1 analogs (exendin), and may not be relevant to potential changes that could occur with long-term exposure. A human study of type 2 diabetic patients administered liraglutide for 12 weeks found a reduction in fasting glucagon with all 5 liraglutide doses tested, although statistical significance was not reached [4].

Importantly, a recent article describing the effects of long-term exposure to GLP determined that there was greater α-cell mass in humans treated with incretins for more than 1 year compared to diabetics on other drugs [31]. The subjects on the incretins had expanded numbers of glucagon-positive cells lining the pancreatic ducts associated with increased, but still rare, Ki-67 staining. The results of this study are at odds with our findings. However, it is important to note that the human organ donors studied in Butler et al. were taking sitagliptin, a DPP-4 inhibitor ($n = 6$) and one was on exenatide. The differences between our findings and the human study may certainly be due to the aspects unique to ZDF rats, or may be drug specific as we only tested the effects of liraglutide. Further studies are vital to determine the long-term effects of drugs within the incretin classification on α-cell health and pancreatic function.

Other human studies on the long-term effects of GLP-1 analogs have begun to raise concerns about the safety of this class of drugs [32,33]. While the majority of the studies have examined possible links between the use of another GLP-1 agonist, exendin, likely because it has been on the market longer, new reports including case studies, indicate a possible link with liraglutide as well [34–36]. In the past 2 years, the FDA has issued warnings concerning the use of liraglutide with possible risks of thyroid cancer, pancreatitis, and lost renal function [37]. Although studying these complications was not the focus of this study, no obvious signs of pancreatitis were noted in the liraglutide-treated animals.

5. Conclusions

This study, along with previous publications, showed that liraglutide had positive metabolic effects on the ZDF rat model, including reduced weight gain and decreased non-fasting blood glucose levels. However, this study also demonstrates abnormal islet morphology associated with severe diabetes that was not reversed by liraglutide and, in the case of α-cell health, was actually inferior in the treated group. Any hope of stabilizing or enhancing islet cell mass or function must be accompanied by studies that carefully assess islet anatomy and physiology. The relevance of these findings to humans needs to be investigated, along with investigations of the mechanism of α-cell loss.

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Conflict of interest

The authors have no conflict of interest with this project.

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