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GLP-1-related proteins attenuate the effects of mitochondrial membrane damage in pancreatic β cells

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ABSTRACT

Glucagon-like peptide (GLP)-1 analog based therapies are used not only for their insulinotropic effects, but also for their pleiotropic effects that improve pancreatic β cell function. Liraglutide is a long acting derivative of human GLP-1(7–37), which is a cleavage product encompassing amino acids 7–37 of GLP-1. In this study, we examined whether Liraglutide treatment restore the glucose-stimulated mitochondrial response of β cells with chemically induced mitochondrial damage. We tested three GLP-1-related proteins: human GLP-1(1–37), GLP-1(7–37) and Liraglutide. To measure changes of the mitochondrial pH quantitatively in real-time, we have developed a bioengineered β cell line. We generated a mitochondrial damaged model by treating β cells with ethidium bromide (EtBr; 0.5 or 1 μg/mL for 48 h). EtBr treatment reduced the response to 25 mM glucose in mitochondrial pH in a dose- and time-dependent manner. GLP-1(7–37) (100 nM) enhanced the response of mitochondria to glucose stimulation in undamaged β cells. Preincubation with Liraglutide (1 nM) or GLP-1 (100 nM) for 3 h recovered the mitochondrial response to glucose in damaged β cells, however, GLP-1(7–37) (100 nM) did not. When GLP-1(7–37) was administered in stepwise increments (i.e., starting with 20 nM to reach 100 nM in 3 h), similar recovery of the mitochondrial function was observed. The results suggest that Liraglutide is effective to recover glucose-stimulated mitochondrial response in damaged β cells.

1. Introduction

The glucagon-like peptide-1 (GLP-1) analog and inhibitor of its cleavage enzyme dipeptidyl peptidase-4 (DPP-4) are demonstrated to have insulinotropic effects as well as pleiotropic effects in patients and mouse models of diabetes [1,2]. Liraglutide is an analogue of GLP-1(7–37) with an added fatty acid to increase solubility and prolong its half-life in plasma through albumin binding. GLP-1(7–37), a peptide generated by proteolysis of the N-terminus of GLP-1, possesses a strong insulinotropic effect [3,4]. The DPP-4 inhibitors prolong a half-life of endogenously released active GLP-1, GLP-1(7–37) as well as glucose-dependent insulinotropic polypeptide (GIP). GIP has been reported to be inactive in diabetic patients [5–7], thus GLP-1 related medicaments have been considered as a therapeutic regimen for treating diabetic patients. GLP-1 analogs were also shown to prevent apoptosis in vivo [8,9], in which restoration of mitochondrial membrane damage may be involved [10]. Liu et al. have shown that the active cleavage product of GLP-1 rescued mitochondrial function [10,11], however, it is not clear whether the mitochondrial response to insulin secretion is recovered. In the glucose tolerance test, obese individuals without liver fat and who retained insulin sensitivity, did not show alterations in GLP-1 secretion, whereas obese subjects with liver fat showed reduction in GLP-1 secretion compared to the prevailing glucose concentration in the test [12]. Both obese and non-obese subjects had similar GLP-1/glucose AUC ratios. However, it is well known that obese individuals and patients with early-stage type 2 diabetes are hyper-insulinemia, thus, we hypothesized that the blunted phase of GLP-1 secretion for 2–3 h in these patients may be required to rescue mitochondrial function in damaged β cells, and further Liraglutide may work effectively, because it has prolonged effects than natural GLP-1.

Mitochondrial metabolism regulates the maximal limit of insulin secretion in cultured β cells [13]. Therefore, the ability of β cells to secrete insulin can be accurately measured by examining mitochondrial function. We have engineered an innovative, chloride-insensitive, hydrogen-sensitive, fluorescent biosensor (MitpHGV) that was used to generate a pancreatic β cell line to monitor mitochondrial pH (MitpHGV-MIN6) [14]. This biosensor is not affected by expression levels of the probe or photobleaching. This β cell line enables us to reliably study the response of mitochondria to glucose stimulation by monitoring changes in mitochondrial pH.

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Ethidium bromide (EtBr) is an inhibitor of DNA and RNA synthesis that, at low doses, selectively inhibits mitochondrial DNA transcription in β cells [15–17]. The β cells that have been depleted of mitochondrial DNA by treatment with a low-dose of EtBr serve as a model of maternally inherited diabetes and deafness (MIDD) [18]. In this study, we used a new fluorescent biosensor to quantitatively examine the mitochondrial response to glucose stimulation in β-cells with EtBr-induced mitochondrial damage. We show that Liraglutide, a GLP-1-related protein, efficiently promotes recovery from mitochondrial damage.

2. Materials and methods

2.1. Measurement of mitochondrial pH and Ca^{2+} signaling in MIN6 cells

MIN6 cells (a kind gift from Prof. Miyazaki) were selected based on Ca^{2+} activity in response to glucose stimulated insulin secretion (25 mM), and stably transfected with *MitpHGV* which is a chloride-insensitive hydrogen-sensitive biosensor probe fused with mitochondrial localization signal as previously described [14]. For dual excitation, we used 380 ± 10 and 480 ± 10 nM interference filters (Omega Optical, Brattleboro, VT) and a single 520 ± 20 nM filter for emission. A 505 DCLP dichroic mirror (Omega Optical) was also used. Images were processed using MetaFlow software (Meta Flow, Tokyo, Japan). Regions of interest were manually selected and pixel intensities were spatially averaged after background subtraction. When bound to DNA, EtBr is a fluorogenic substrate, but it emits fluorescence at a higher wavelength than the one we use to measure pH. Moreover, we used a dichroic mirror to filter out any EtBr fluorescence that might persist after washing the MitpHGV-MIN6 cells. Therefore, the amount of EtBr background fluorescence did not affect the mitochondrial pH measurements.

To measure Ca^{2+} in MitpHGV-MIN6 cells, the cells were incubated with 2 μM Fura-2AM (Dojindo Co. Ltd., Kumamoto, Japan), and Ca^{2+} signals were measured as previously reported [19].

2.2. Effects of GLP-1, GLP-1(7–37), and Liraglutide on mitochondrial pH in glucose-stimulated MitpHGV-MIN6 cells

We generated a model of mitochondrial damage using MitpHGV-MIN6 β-cells by treating cells with 0.5, 1, or 10 μg/mL EtBr (Sigma Aldrich Co., MO, USA) for 24 or 48 h.

GLP-1 and GLP-1(7–37) were purchased from Sigma Aldrich and Liraglutide was provided from Novo Nordisk A/S (Bagsvaerd, Denmark).

2.3. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Data and curves were averaged using Igor pro (WaveMetrics, Inc.,

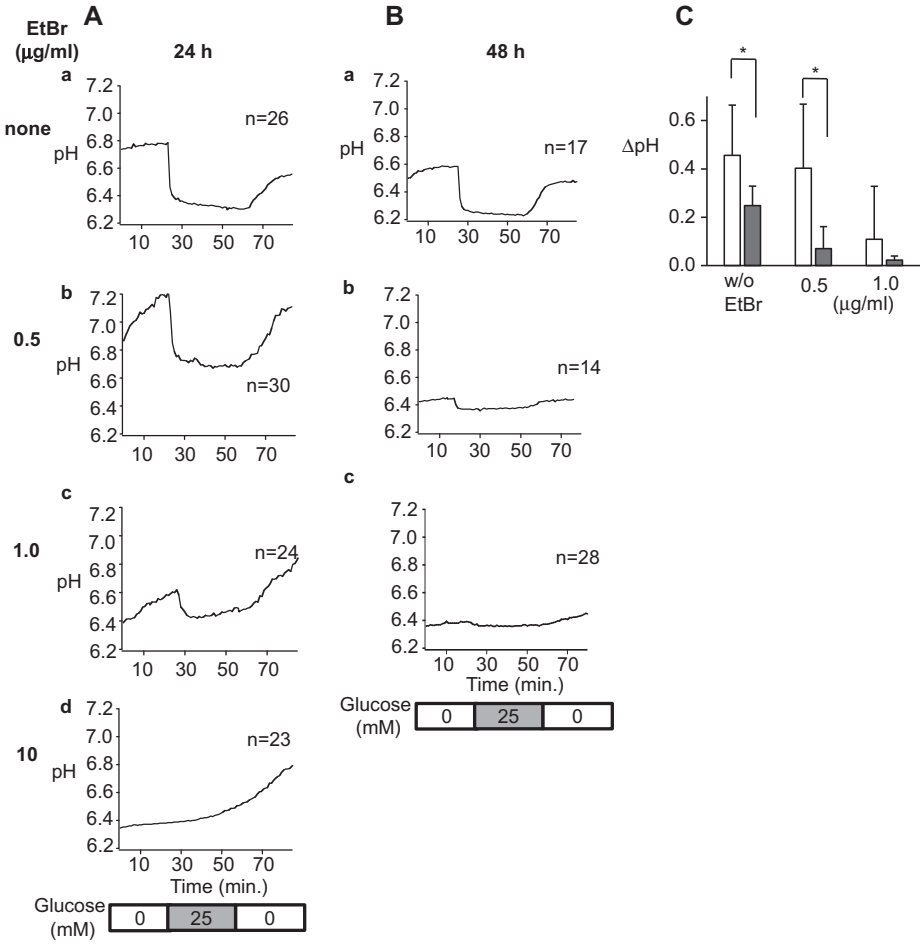

Fig. 1. Quantitative assessment of mitochondrial membrane function in damaged MitpHGV-MIN6 cells. EtBr reduced glucose-stimulated (25 mM) acidification of the mitochondrial intermembrane space in a dose- and time-dependent manner. (A) Exposure of MitpHGV-MIN6 cells to EtBr for 24 h. The response of mitochondrial pH to 25 mM glucose stimulation in the absence of EtBr (a) and in the presence of 0.5 μg/mL (b), 1 μg/mL (c) and 10 μg/mL EtBr (d). (B) Exposure of MitpHGV-MIN6 cells to EtBr for 48 h. The response of mitochondrial pH to 25 mM glucose stimulation in 24 h after the measurement in A (a). The mitochondrial pH response in the presence of 0.5 μg/mL (b) and 1 μg/mL EtBr (c). (C) The glucose-stimulated change in mitochondrial pH (ΔpH) in MitpHGV-MIN6 cells exposed to EtBr for 24 h (white column) and 48 h (black column).

Oregon, USA) from over 20 data sets. ΔpH indicates differences in mitochondrial pH in the presence and absence of glucose.

3. Results

3.1. A β cell model of mitochondrial damage

Mitochondrial membrane pH was decreased by 0.46 ± 0.18 along with 25 mM glucose load (Fig. 1A-a). EtBr pretreatment for 24 h reduced intermembrane acidification in response to glucose stimulation (25 mM) dose-dependently (ΔpH 0.40 ± 0.27 with EtBr 0.5 μM and 0.11 ± 0.22 with EtBr 1.0 μM) (Fig. 1A-b,c), whereas 10 μg/mL EtBr abolished the response completely (Fig. 1A-d). Further reduction of intermembrane acidification was observed by 48 h pretreatment of EtBr (ΔpH; 0.07 ± 0.09 with EtBr 0.5 μM and 0.02 ± 0.01 with EtBr 1.0 μM) (Fig. 1B). Mitochondrial responses by 0.5 μM EtBr were significantly decreased by 48 h incubation compared to 24 h ($p < 0.01$). No significant difference was observed between 48 h and 24 h incubation with 1.0 μM EtBr (Fig. 1C). Note that Ca^{2+} signals were not affected in these cells (data not shown), indicating that the damage in this β cell model was mitochondria-specific.

3.2. Acute administration of GLP-1(7–37) did not change the mitochondrial response to glucose stimulation in mitochondria damaged β cells

The acute effect of GLP-1-related proteins on the mitochondrial pH of glucose-stimulated β cells was observed by 30 min pre-incubation with GLP-1(7–37) before stimulation with glucose. In control cells, while there was no change in the mitochondrial pH on loading 100 nM GLP-1(7–37) without glucose, treatment with 100 nM GLP-1(7–37) increased the mitochondrial pH(ΔpH; 0.44 ± 0.10, 1.35 ± 0.83 without and with GLP-1(7–37), respectively) and Ca^{2+} signaling responses (data not shown) induced by glucose stimulation. (Fig. 2A-a,d)

The acute effect of GLP-1-related proteins on the glucose-stimulated mitochondrial pH was observed by 30 min pre-incubation prior to glucose. In control cells, while there was no

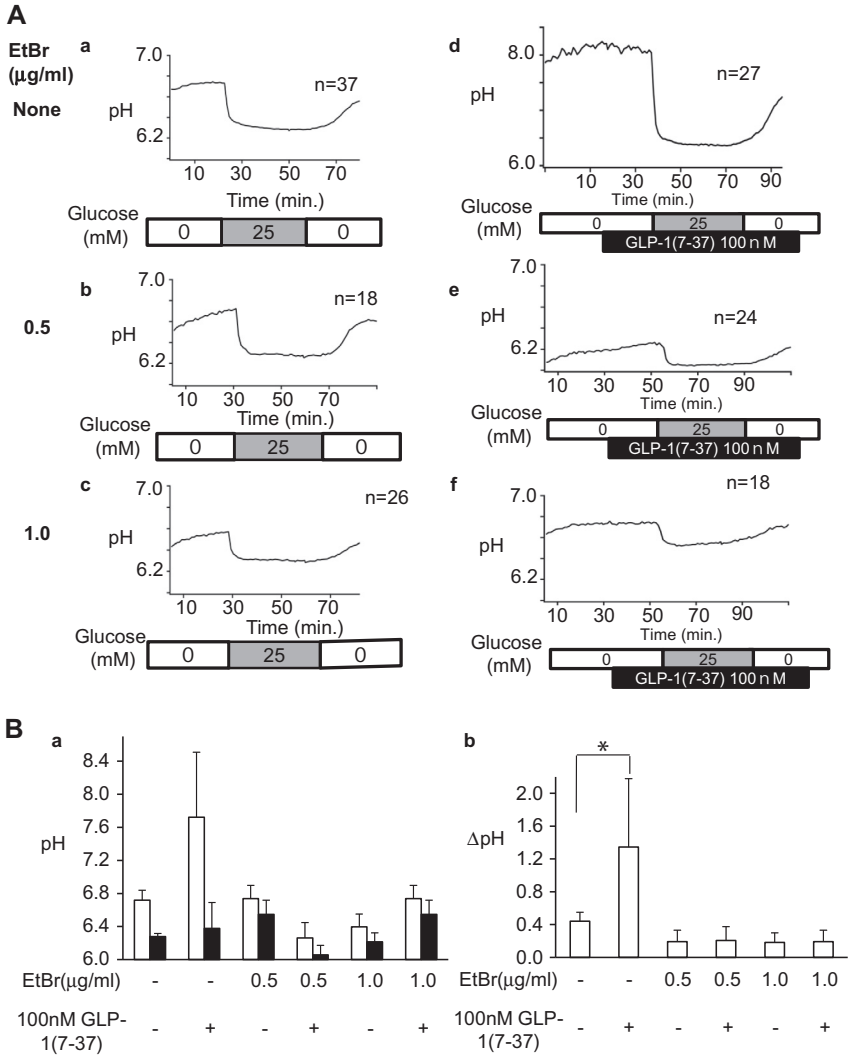

Fig. 2. The effect of GLP-1(7–37) on the glucose-stimulated (25 mM) mitochondrial pH response in MitpHGV-MIN6 cells in the presence or absence of EtBr-induced mitochondrial damage. (A) The effect of GLP-1(7–37) administration on mitochondrial pH in MitpHGV-MIN6 cells. The mitochondrial pH in cells without EtBr-induced damage (a) and cells exposed to 0.5 μg/mL (b) and 1.0 μg/mL EtBr for 48 h (c). The mitochondrial pH in undamaged cells (d), cells exposed to 0.5 μg/mL (e) and 1.0 μg/mL EtBr for 48 h acutely treated with GLP-1(7–37). (B) The effect of GLP-1(7–37) administration on mitochondrial pH in the presence (black column) and absence (white column) of 25 mM glucose in MitpHGV-MIN6 cells with or without EtBr damage (a). The effect of GLP-1(7–37) on mitochondrial pH change in the presence or absence of glucose stimulation (b). Bars represent standard deviation (SD).

change in mitochondrial pH without glucose, 100 nM GLP-1(7–37) increased the mitochondrial pH (ΔpH; 0.44 ± 0.10, 1.35 ± 0.83 without and with GLP-1(7–37), respectively) (Fig. 2A-a,d) and Ca^{2+} signaling responses (data not shown) induced by glucose stimulation. In EtBr damaged cells, GLP-1(7–37) did not affect the mitochondrial intermembrane pH by 0.5 μM EtBr (ΔpH; 0.19 ± 0.13, 0.20 ± 0.16 without and with GLP-1(7–37), respectively) (Fig. 2A-b,e) and 1.0 μM (ΔpH;0.18 ± 0.12, 0.19 ± 0.13 without and with GLP-1(7–37), respectively) (Fig. 2A-c,f) or Ca^{2+} signaling response (data not shown), when glucose was administered simultaneously. Neither GLP-1 (100 nM or 500 nM) nor Liraglutide (10 nM) treatment elicited changes in mitochondrial membrane pH in undamaged and damaged β cells (data not shown).

3.3. Recovery from mitochondrial damage in β-cells preincubated with GLP-1 or Liraglutide

In EtBr damaged cells, 3 h preincubation with GLP-1(7–37) (100 nM) showed no effect on mitochondrial function treated with 0.5 μM EtBr (ΔpH; 29.2 ± 24.5% and 26.1 ± 7.4%, without and with GLP-1(7–37), respectively) and 1.0 μM EtBr (ΔpH;14.5 ± 18.2% and 15.3 ± 16.5%, without and with GLP-1(7–37), respectively) (Fig. 3A-a). Note that data indicate the percent of responses in EtBr-treated cells normalized to untreated control cells. Co-treatment with DPP-4 inhibitor and GLP-1(7–37) also did not elicit any change in the mitochondrial response (data not shown).

Since co-administration of GLP-1 (100 nM) and glucose had no effect, effects of GLP-1 were examined by preincubating the cells with GLP-1 for 3 h. GLP-1 was washed out prior to glucose stimulation. Preincubation of damaged cells (0.5 μg/mL EtBr for 48 h) with 100 nM GLP-1 reversed the attenuated mitochondrial response ($p < 0.01$, ΔpH; 0.12 ± 0.1 and 0.37 ± 0.07, without and with GLP-1, respectively) (Fig. 3A-b). With higher concentration of EtBr (1.0 μg/mL), however, the mitochondrial response was not reversed (ΔpH; 0.06 ± 0.08 and 0.07 ± 0.07, without and with GLP-1, respectively). A lower concentration of GLP-1 (10 nM) showed no effect on cells treated with 0.5 μg/mL EtBr (data not shown).

Consistent with the effect of 100 nM GLP-1, 10 nM Liraglutide reversed the damage induced by 0.5 μg/mL EtBr ($p < 0.01$, ΔpH; 30.6 ± 39.3% and 83.9 ± 48.9%, without and with 10 nM Liraglutide, respectively) and 1 μg/mL EtBr ($p < 0.01$, ΔpH; 9.9 ± 7.3 and 24.1 ± 19.9%, without and with 10 nM Liraglutide, respectively). 1.0 nM Liraglutide recovered EtBr damaged cells (0.5 μg/mL:$p < 0.01$, ΔpH; 55.4 ± 13.4 and 136.8 ± 37.7%, without and with 1.0 nM Liraglutide, respectively), but not 1 μg/mL EtBr-treated cells (ΔpH; 8.4 ± 7.4% and 5.9 ± 18.4%, without and with 1.0 nM

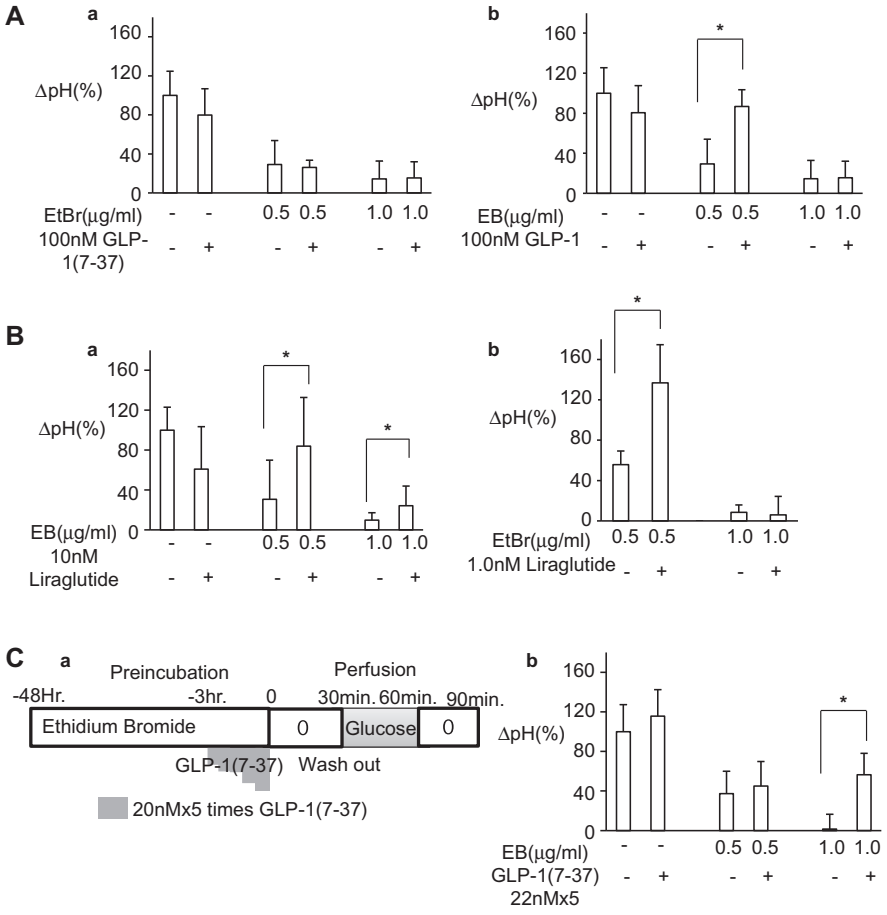

Fig. 3. The effect of GLP-1-related protein pre-incubation on mitochondrial function in MitpHGV-MIN6 cells with or without EtBr-induced mitochondrial damage. Mitochondrial ΔpH due to 25 mM glucose load with and without preloading of GLP-1s, after washout of GLP-1s in EB damaged MIN6 cells. Data indicate the ratio (%) of which compare to MIN6 cells without EtBr-induced mitochondrial damage. The data are all mean value of more than 10 cells. Bars represent SD. (A) (a) The response of mitochondrial pH to glucose stimulation (25 mM) in cells preincubated with GLP-1(7–37) (100 nM) for 3 h. (b) The response of mitochondrial pH to glucose stimulation (25 mM) in cells preincubated with GLP-1 (100 nM) for 3 h. (B) The response of mitochondrial pH to glucose stimulation (25 mM) in cells preincubated with 10 and 1.0 nM Liraglutide for 3 h. Glucose-stimulated mitochondrial ΔpH in MitpHGV-MIN6 cells exposed to 0.5 and 1.0 μg/mL EtBr for 48 h and preincubated with 1.0 nM (a) and 10 nM (b) Liraglutide. (C) (a) Procedure to mimic the chronic effects of GLP-1 and Liraglutide using GLP-1(7–37). The mitochondrial pH was measured in cells preincubated with a total of 100 nM GLP-1(7–37) added to the medium in 5 step-wise increments and stimulated with glucose (25 mM) after GLP-1-related proteins were washed out. (b)The response of mitochondrial pH to glucose stimulation (25 mM) in EtBr-damaged cells (0.5 μg/mL EtBr for 48 h) preincubated with GLP-1(7–37) administered in five increments (20 nM each) for a total of 100 nM GLP-1(7–37) delivered over the course of 3 h.

Liraglutide, respectively) (Fig. 3B-b). Taken together, Liraglutide is more potent in ameliorating mitochondrial damage than GLP-1.

Next, we tested whether a step-wise administration of GLP-1(7–37) would mimic the effect of GLP-1 and Liraglutide, since GLP-1(7–37) has a greater binding affinity to the receptor. GLP-1(7–37) was administered in 5 (20 nM each) increments until reaching100 nM total in 3 h (Fig. 3C-a). It reversed EtBr-induced damage by 1.0 μg/mL EtBr treatment ($p < 0.01$ ΔpH; 1.5 ± 14.7% and 56.5 ± 21.5%, without and with GLP-1(7–37), respectively) (Fig. 3C-b), but not in cells treated with 0.5 μg/mL EtBr-induced damaged cell (ΔpH; 37.3 ± 22.6% and 45.1 ± 24.7%, without and with GLP-1(7–37), respectively).

4. Discussion

Mitochondrial membrane function in the damaged β cells was quantitatively analyzed using our MitpHGV biosensor system. The mitochondrial response to glucose was attenuated in MitpHGV-MIN6 cells with damaged mitochondria. Liraglutide (1 nM and 10 nM) restored the mitochondrial response in β cells in a dose-dependent manner. Liraglutide had no effect on the mitochondrial membrane response in undamaged β cells, which is consistent with the results of clinical studies that suggested that excessive dose of Liraglutide did not induce hypoglycemia [20,21]. Liraglutide binds to albumin, thereby prolonging its half-life in plasma, which results in a slow and consistent release from albumin. Another GLP-1 analog, Lixisenatide, has a shorter half-life than Liraglutide (12-h half-life) and has been reported to decrease postprandial glucose levels more effectively [22]; however, the HbA1c level of patients received Lixisenatide was not found to significantly differ from those received Liraglutide [23]. The present study suggests that long and moderate action of Liraglutide leads to more efficient restoration of mitochondrial function in β cells than the shorter half-life GLP-1 analogs. Liraglutide stimulates insulin secretion from β cells only under hyperglycemic conditions and restores mitochondrial membrane function. GLP-1 did change the mitochondrial membrane response induced by glucose stimulation, only when a high dose was administered (100 nM). GLP-1 has been demonstrated to be secreted by α cells under certain conditions [24,25]. Alpha-cells in human pancreatic islets have also shown to secrete GLP-1, which may be attenuated by type 2 diabetes [26]. Upregulation of GLP-1 may increases β cell mass following a metabolic or inflammatory insult [27]. Disruption of β cell function is accompanied by an increased release of GLP-1, suggesting a paracrine effect of GLP-1 [28]. The secretion pattern of GLP-1(7–37) is two-phased in an oral glucose tolerance test: a spiked phase followed by a second moderate phase, which continues for almost 2 h after glucose load. In the unhealthy pattern seen in obesity, secretion of GLP-1(7–37) decreases in the first phase [12]. We have shown that GLP-1(7–37) secretion at constant levels for 3 h is necessary for restoring the normal mitochondrial response to glucose in β cells with mitochondrial damage. The second phase of GLP-1(7–37) secretion may also be important for recovery from mitochondrial damage in β cells.

In conclusion, GLP-1 analogs may exert clinical effects on β cell function by directly acting on the mitochondrial membrane. Long-acting Liraglutide efficiently recovers mitochondrial damage in β cells.

Acknowledgments

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