MiR-7, miR-9 and miR-375 contribute to effect of Exendin-4 on pancreatic β-cells in high-fat-diet-fed mice

Abstract

Purpose: The purpose of this study was to test whether glucagon-like peptide-1 (GLP-1) receptor activation preserved pancreatic β-cells via the regulation of microRNAs and target genes in high-fat-diet-fed mice.

Methods: C57BL/6 male mice were simultaneously treated with high-fat-diet (HFD) and GLP-1 analogue, Exendin-4 (Ex-4) (3 μg/kg/day or 30 μg/kg/day), i.p. or vehicle, for consecutive 13 weeks. Fasting blood glucose, postprandial blood glucose, ΔI_{30}/ΔG_{30}, HOMA-IR and HOMA-%β were measured in each group. Pancreatic β-cell mass was assessed by immunohistochemistry. The expression of miRNAs and related downstream genes were investigated using quantitative real-time PCR.

Results: Thirteen weeks of Ex-4 treatment significantly reduced body weight and food intake in HFD-fed mice (P<0.05). Insulin sensitivity, HOMA-IR and HOMA-%β were markedly improved in Ex-4-treated groups (P<0.05). Histological examination revealed that β-cell mass was significantly increased in high dose Ex-4-treated mice (P<0.05). Interestingly, Ex-4-treated islets displayed significant down-regulation of the expression of miR-7, miR-9 and miR-375 and up-regulation of the levels of mammalian target of rapamycin (mTOR), one cut homeobox 2 (OC-2) and phosphoinositide-dependent protein kinase-1 (P<0.05).

Conclusion: MicroRNAs and genes targeted in response to GLP-1 receptor agonism were involved in preserving β-cell mass and function in HFD-induced mice; which suggest a mechanism involving the GLP-1 receptor as a therapeutic approach for the treatment of type 2 diabetes mellitus and obesity. This study also shows the potential for elucidating other important therapeutic targets for diabetes.
Type 2 diabetes mellitus (T2DM) is characterized by defective insulin secretion and insulin resistance, leading to hyperglycemia. This defect in insulin secretion is accompanied by a deficit in β-cell mass [1,2]. Recently, a glucagon-like peptide-1 (GLP-1) mimetic class of drugs, widely referred to as incretins, has shown promise as a therapeutic for treatment of T2DM [3,4]. A growing body of evidence has shown that GLP-1 can enhance glucose-stimulated insulin secretion (GSIS) and insulin synthesis, inhibit glucagon secretion, confer glucose sensitivity to glucose-resistant β-cell, stimulate β-cell proliferation and neogenesis and inhibit β-cell apoptosis [5-7]. In addition, exogenous GLP-1 administration inhibits gastric emptying and appetite, potentially leading to weight loss [8]. Based on these observations, GLP-1R activation has become an attractive therapeutic target for T2DM.

**MicroRNAs (miRNAs)** are a class of short, single-stranded non-protein coding gene products that regulate gene expression through post-transcriptional inhibition of mRNA translation [9]. MiRNAs play a key role in a diverse set of physiological processes that dysregulating the expression of miRNAs, leading to biochemical dysfunction and subsequently to the onset of diseases such as T2DM [10,11]. There is growing evidence that a large number of miRNAs play significant roles in insulin production, secretion and function, as well as in the maintenance of pancreatic islet phenotypes, proliferation, development and apoptosis, so close attention has been paid to miRNAs in T2DM treatment [12-16].

In recent years, studies have indicated that miRNAs are involved in effects on pancreatic cells; for example, it has been shown that miR-34a, and its downstream gene SIRT1, contributed to the protective effects of GLP-1 against lipotoxicity in vitro [17]. Previous studies have also shown that miR-7-mediated down-regulation of mTOR led to the reduction of β-cell proliferation [18, 19]. MiR-375 interacted with phosphoinositide-dependent protein kinase-1 (PDK-1) mRNA resulted in decreased glucose-stimulatory action on insulin gene expression and synthesis [20,21]. MiR-9-mediated down-regulation in the expression of transcription factor OC-2 has been shown to lead to the impairment in GSIS (13, 22). Together, these findings suggested a close relationship between microRNAs and β-cell development, proliferation and function; however, there is no direct evidence to show whether, and the mechanism by which, microRNAs, particularly miR-375, miR-9 and miR-7, play a role in GLP-1-induced beneficial effects.

We hypothesized that miRNAs, and their related genes, contribute to the potential effects of GLP-1R agonist—preserving β-cell mass and function. To test this hypothesis, a set of C57BL/6 male mice were fed with a high-fat-diet (HFD) at 5 weeks of age. Simultaneously, Exendin-4 (Ex-4) was administered intraperitoneally for 13 weeks. Whole body metabolic profiles, as well as β-cell function and proliferation, were assessed. Underlying mechanisms were also explored by investigating expression levels of miRNAs (miR-375, miR-9 and miR-7) and their closely related genes (PDK1, One-cut-2 and mTOR) in these mice.

**Methods**

**Animals and diets**

Four-week-old male C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China) and acclimated for 7 days before the experiments were initiated. Animals were housed individually (21–24°C, humidity 30-70%, 12:12-hour light cycle) with ad libitum access to water and a HFD with 60% of calories derived from fat (D12492) (Research Diets, New Brunswick, NJ, USA). Food and water intake were measured each week and at the termination of each study. Mice were weighed weekly so that drug doses could be adjusted. All experiments were approved by the Institutional Animal Care and Use Committee at Laboratory Animal Center of Second Affiliated Hospital of Harbin Medical University.

**Generation of a diet induced obesity model and study design**

All mice were fed on HFD for 10 weeks and oral glucose tolerance tests (OGTTs) were performed [23]. Individual mice with hyperglycemia (fasting blood glucose ≥ 145 mg/dL, blood glucose at 2 hours post-oral glucose challenge ≥ 300 mg/dL) for three consecutive days were used as obesity mouse model in later experiments.

Exendin-4 (Bachem, Switzerland) was dissolved in PBS for injection. Age- and weight-matched mice were randomly allocated to three study groups using a computer-based randomization. Three groups of mice were studied: 1) HFD-fed control; 2) Ex-4 low dose (3 μg/kg day); and 3) Ex-4 high dose (30 μg/kg day). In the two treatment groups (Ex-4, 3 and 30 μg/kg), Ex-4 was administered via i.p. infusion twice daily (at 0800 h and 1800 h) for 13 weeks, while the control groups were treated with saline for the same duration. Ex-4 treatment animals were dose-titrated during the first 7 days to avoid adipsia-induced dehydration.
Clinical chemistry

After 13 weeks of Ex-4 treatment, 8 h-fasted mice were given an oral bolus of 3 g/kg D-glucose (Amresco, Solon, OH, USA) for OGTTs. Blood samples were collected by tail vein or retro-orbital sinus puncture at 0, 30 and 120 min after glucose administration. Blood glucose concentration was determined using the On-Touch ultra blood glucose meter (LifeScan, Milpitas, CA, USA). Following centrifugation at 4°C, serum was separated and stored at -80°C until further analysis. Serum insulin was assessed with ELISA kits according to assay protocol (mouse insulin ELISA, Merodia, Uppsala, Sweden). Pancreatic β-cell function was calculated as the difference in values between 0 and 30 min using the following equation: ΔG30/ΔG30 = (I30-I0) / (G30-G0). HOMA-IR and HOMA-%β were calculated as described [24] by using the equations: HOMA-IR = (FPG × FPI)/22.5. HOMA-%β = (FPI × 20)/ (FPG - 3.5). FPI is fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/L).

Histology

All animals were anesthetized with CO2 before decapitation. The pancreas was removed, weighed and immediately fixed in 4% buffered formaldehyde. Pancreatic tissue was dehydrated and paraffin embedded according to standard histological procedures. Sections (5 μm) were cut and stained with hematoxylin-eosin. The sections were incubated with rabbit anti-insulin polyclonal antibody (Bioss, 1:400). The secondary antibody labeled with peroxides (Boster, Wuhan, China). The sections were then stained with 3,3-diaminobenzidine (DAB, Beijing Zhong Shan Biotech, Beijing, China) and nuclei counterstained with hematoxylin. Relative β-cell area was calculated by percentage of the positive insulin area in the total pancreas area of each section. The β-cell mass was measured by first quantifying relative β-cell area and multiplying this by the pancreatic weight [25]. Relative β-cell mass was calculated as total β-cell mass divided by body weight. The slides were imaged using Nikon DS Ri1 (Nikon, Tokyo, Japan) and analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Quantitative real-time PCR

RNA was extracted from pancreatic tissue using an RNA extraction kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s instructions. The miRNA was reverse transcribed using a miRcute miRNA First-Strand cDNA Synthesis kit and detected using a miRcute miRNA qPCR Detection kit (TIANGEN BIOTECH, Beijing, China). The RNA was converted to cDNA using a Quant First-Strand cDNA Synthesis kit and detected using a RealMaster Mix Detection kit (TIANGEN BIOTECH). The PCR products were fluorometrically quantified using SYBR Green, normalized to a housekeeping gene (5S or GAPDH) and expressed relative to the control. Analysis of real-time PCR data was evaluated using the 2^ΔΔCt method.

Statistical analysis

Data are presented as means ± SEM. All results are graphed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical differences between treatment groups and appropriate controls were assessed with one-way ANOVA followed by Bonferroni’s multiple comparison test using SPSS.18. Statistical significance was set at P<0.05.

Results

Treatment with Ex-4 significantly reduces body weight and food consumed in HFD-fed mice

As shown in Figure 1, mice that were HFD-fed gradually increased their body weights. While average body weights in low and high dosed Ex-4 treatment groups were significantly lower than the HFD control group after just 1 week of Ex-4 treatment, and the differences were sustained throughout the rest of experimental period (P<0.01, Figure 1A). In addition, there was a significant reduction in food intake in both Ex-4 treatment groups compared with the HFD control group (P<0.05, Figure 1B).

Treatment with Ex-4 improves glucose tolerance and insulin responsiveness in HFD-fed mice

To test whether treatment with Ex-4 could modulate glucose tolerance and insulin sensitivity in mice, C57BL/6 mice fed with HFD for 10 weeks were divided into two groups and fed with HFD alone or HFD with Ex-4 (3 and 30 μg/kg day) for an additional 13 weeks. Fasting blood glucose levels, glucose tolerance, ΔI30/ΔG30, HOMA-%β and HOMA-IR index were measured (Figure 2). ΔI30/ΔG30, the insulinogenic index, was significantly enhanced in both low- and high-dose Ex-4 treatment groups in relative the HFD control group (P<0.01, Fig. 2A). Moreover, the HOMA-IR index, an indicator of insulin resistance, was notably reduced in the two Ex-4 treatment groups relative to the HFD control group (P<0.01, Fig. 2B). Mouse treated with high-dose Ex-4 exhibited...
significant increase in HOMA-%β compared with the HFD control group (P<0.01, Figure 2C). There was no difference between low- and high-dose Ex-4 treatment mice in the values of ΔI30/ΔG30, HOMA-IR and HOMA-%β.

**Effects on morphology of pancreatic islets and beta-cell mass**

To understand the mechanisms underlying the improved glucose tolerance by Ex-4 under HFD condition, the morphology of pancreatic islets were assessed (Figure 3A-3C). There was no gross abnormality of islets observed in two Ex-4 treated mice, other than a little more scattered single and small clusters of insulin-positive cells in the high-dose Ex-4-treated group (Figure 3D). The relative β-cell mass (% body weight) in high-dose Ex-4 mice was significantly increased compared with the HFD-fed mice (P<0.05, Figure 3E), while the relative β-cell mass of the low-dose Ex-4 was comparable with the HFD-fed mice. Meanwhile, there was no statistical difference between high- and low- dose Ex-4 groups with regards to β-cell mass.

**Effects of Ex-4 on expression of microRNAs and related target genes**

A close relationship between microRNAs, particularly miR-7, miR-9 and miR-375, and β-cell proliferation and insulin secretion has been suggested. The levels of miRNA (miR-7, miR-9 and miR-375) and related target genes (mTOR, OC-2 and PDK1) in HFD-induced mice in the presence or absence of Ex-4 are shown in Figure 4. The level of miR-7 showed a sharply decrease in the high-dose Ex-4 treatment group compared with the HFD control group (Figure 4A, P<0.05). High-dose Ex-4 significantly increased the expression of mTOR compared to HFD control group (Fig. 4D, P<0.05), which with coincided with the reduction of miR-7 level. There was no difference in the levels of miR-7 and mTOR between the low dose Ex-4 and HFD control groups. Consistent with serum biochemical studies of β-cell function, the expression of miR-9 and miR-375 was dramatically reduced in both of high- and low-dose Ex-4 groups, compared with the HFD control group (Figure 4B and 4C, P<0.01). Furthermore, both 13-week low-and high-dose Ex-4 treatment exhibited a statistically significant increase in the transcript levels of one cut homeobox 2 (OC-2) and PDK-1 compared with the HFD control mice (Fig. 4E and 4F, P<0.01). In addition, there was no significant difference between low- and high-dose Ex-4 treated groups in the expression of above mentioned six genes. In other words, there is no dose-dependent response observed.

**Discussion**

Exendin-4 is the first GLP-1R agonist to be approved for human clinical use in the USA in April 2005. It was originally isolated from *Heloderma suspectum* saliva, which shares 53% amino acid sequence with human GLP-1 [26]. Ex-4 has a
much longer half-life in vivo, which allows us to examine the in vivo effects of GLP-1 signalling. In the present study, the results demonstrated that the intraperitoneal twice-daily administration of Ex-4, at doses of 3 and 30 μg/kg/day for 13 weeks in high-fat diet-induced mice, decreased body weight and blood glucose, improved insulin resistance and preserved β-cell mass. Furthermore, we investigated the potential molecular mechanisms underlying preservation of pancreatic function of Ex-4. Our results revealed that the specific microRNAs (miR-7, miR-9, and miR-375) and their target genes (mTOR, OC-2, and PDK-1) contributed to GLP-1 regulating β-cell preservation.

Previous studies have shown that Ex-4 regulates glucose metabolism and reduces body weight and food intake [27-29]. In this study, OGTT and insulin release test revealed that Ex-4, at a dose of 30 μg/kg/day, promoted whole body glucose stimulated insulin secretion and improved insulin resistance as determined by calculating HOMA-IR and HOMA-%β. The value of HOMA-IR was decreased in both dose of Ex-4 group compared with the control group; probably because Ex-4 treatment decreases blood glucose and improves insulin responsiveness, which contributes to reduced demand for insulin content and β-cell growth, and finally leads to a decrease in the value of HOMA-IR as demonstrated in previous study [30]. A high dose of Ex-4 could significantly reduce HFD-induced aggravation of insulin resistance to a normal level, while a low dose of Ex-4 was unable to do this. Although it failed to achieve significant, the value of HOMA-%β was trending upwards in low-dose Ex-4 towards HFD. The failure in normalization of HOMA-%β in low-dose Ex-4 group may have been partly due to the insufficiency in effective body weight and food intake control, which showed a little bounce back after 8 weeks exposure to Ex-4.

In this study, a significant increase in β-cell mass divided body weight was observed in the Ex-4 high dose group, which reflected a potential role of Ex-4 in enlargement of β-cell mass. It is consistent with previous studies that have demonstrated an increase in β-cell mass and proliferation in diabetic animals after Ex-4 treatment [27, 31]. There was no significant difference in the β-cell mass between low-dose Ex-4 group and...
HFD-fed group. Thus, a dose-dependent response observed in the effect of Ex-4 on β-cell mass. The improvement of glucose control and insulin resistant in both Ex-4 groups suggested that the improvement in glucose metabolism under Ex-4 administration was not necessarily fully dependent on an increase in β-cell mass. Ex-4 treatment ameliorated insulin resistance, improved efficiency of insulin secretion and function and reduced demand for insulin content and β-cell mass, as was demonstrated in a previous study [30].

A series of studies have found that dysregulation of miRNAs is associated with insulin resistance and diabetes [32]. Ex-4 is widely known to enhance glucose-stimulated insulin secretion (GSIS) and insulin synthesis, confer glucose sensitivity to glucose-resistant β-cell, and stimulate β-cell proliferation and neogenesis. Ex-4 and some miRNAs both target β-cell, but their potential links were not fully clear. Our previous study has demonstrated that miR-34a and its downstream gene, SIRT1, contributed to the protective effects of GLP-1 against lipotoxicity in vitro [17]. Furthermore, our earlier study showed that GLP-1 contributed to increased PGC1α expression by downregulating miR-23a to reduce apoptosis [33]. Similarly, a recent study showed that downregulation of miR-139-5p contributed to the antiapoptotic effect of liraglutide on the diabetic rat pancreas and INS-1 cells by targeting IRS1 [34].

In this study, we measured the expression of some miRNAs and their known target genes after Ex-4 treatment. MiR-7, a representative islet miRNA, dynamically adjusts its
expression across the process of pancreas formation and development [35] and functions as a negative regulator of β-cell proliferation [19]. Our results demonstrated that a high dose of Ex-4 repressed the expression of miR-7 in parallel with an increase in the level of mTOR. Considering these data in the context of previous reports, we hypothesized that miR-7 and its downstream gene mTOR were involved in Ex-4-induced β-cell proliferation. In the study, a decrease in miR-7 as well as an increase in mTOR was observed in response to high-dose of Ex-4, while no significant change was seen with low-dose Ex-4. These observations correspond to the immunohistochemistry results that show that the high-dose Ex-4 group exhibited an increase in β-cell mass; therefore, changes in the expression of miR-7 could lead to changes in beta-cell mass.

MiR-375 directly interacted with the 3-UTR of PDK1 mRNA, resulting in decreasing glucose-stimulatory action on insulin gene expression and DNA synthesis and GSIS [20, 21]. Our results showed that 13 weeks of Ex-4 administration (either low- or high-dose) down-regulated miR-375 and the expression of PDK-1 compared with HFD group. This suggests the repression of miR-375 and stimulation of PDK-1 by Ex-4 may play a role in β-cell preservation and represents another way to enhance insulin release.

Additionally, in present study, up-regulated increase in the expression of OC-2 in both Ex-4-treated groups coincided with a decrease in the expression of miR-9. This observation suggested the potential of Ex-4 to regulate the expression of miR-9 and its downstream gene OC-2, which ultimately resulted in an improvement in GSIS and β-cell function. This in agreement with the insulin secretion pattern. Thus, the expression of miR-375 and miR-9 could affect insulin gene expression and insulin secretion, and further influence metabolic index ∆I30/∆G30, HOMA-IR and HOMA-%β.

A recent study has observed that Sirtuin 1 (SIRT1), a positive regulator of insulin secretion and another target of miR-9 [22], showed altered expression in insulin-secreting β-islets during GSIS in vivo. Based on our observations and previously published reports, it is evident that miR-9 plays an essential role in Ex-4-induced GSIS via different target genes, including OC-2 and SIRT1 and possibly other unknown downstream genes. These observations are consistent with the notion that each single miRNA has the potential to regulate numerous mRNAs, some of which hold more than one miRNA-binding site. Collectively, the protective effects of GLP-1 receptor agonism by Ex-4 on preserving β-cell mass and function was unlikely to be due to the alteration of a single miRNA but to a complex combination of various signalling pathways that have effects on both cell proliferation and function. These findings extend the understanding of mechanisms of GLP and further studies are warranted to delineate the mechanism in great detail.

In conclusion, this study confirmed the effectiveness of Ex-4 on glucose metabolism and suggested that miR-7, miR-9 and miR-375 contribute to the protective effects of Ex-4. These findings may lead to novel therapeutic approaches to improve β-cell function and enhance β-cell mass, which could delay the progression of diabetes.

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References


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