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GLP-1 analogue semaglutide regulates pancreatic beta-cell proliferation via PDX-1 expression control

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Abstract. Semaglutide as an agonist of glucagon-like peptide-1 receptor was shown to potentiate insulin release and suppress food motivation targeting pancreatic islet beta cells and brain region including subfornical organ and hypothalamus, which effectively treats type 2 diabetes and obesity. The investigation of prolonged response with semaglutide on beta cells triggering proliferation and apoptotic resistance is yet to be confirmed. Previous research findings have shown that glucagon-like peptide-1 and liraglutide as agonists for glucagon-like peptide-1 receptors on beta cells result in proliferation through upregulation of PDX-1 transcription factor. Semaglutide is investigated to show whether long-term beta cell survival regulation is achieved through the same downstream signalling pathways. Both in vivo and in vitro methods were designed to show the proliferation effect of the semaglutide in C57BL/6 mice using tissue imaging, cell counting and immunosorbent assay for quantitative analysis of PDX-1 expression. The proliferation effect would broaden the application of semaglutide from insulin augmentation to the sustained benefit of beta cell survival.

Keywords: glucagon-like peptide-1; diabetes; beta-cell; PDX-1

1. Introduction

Semaglutide is a glucagon-like peptide-1 (GLP-1) analogue which acts as an agonist targeting GLP-1 receptors. The GLP-1R were discovered on the pancreatic islet beta cells and within brain in the subfornical organ and hypothalamus [1]. GLP-1, a 30-amino acid peptide produced in the intestinal epithelial endocrine L-cells, is a gut-derived incretin hormone that regulates insulin secretion in a glucose-dependent manner in beta cells. Its action on GLP-1R also results in a prolonged response with activation of genetic transcription involved in proliferation and apoptotic resistance in islet beta cells. Endogenous GLP-1 was shown to induce the proliferation of rat primary islet cells and b-cell lines [2]. And liraglutide, which also acts as an agonist for the GLP-1R, showed increasing proliferation of beta

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cells after alloxan injection during chronic liraglutide treatment [3,4]. This broadens the application of GLP-1 agonists from potentiating insulin secretion to promoting the retention and proliferation of beta cells through the translocation of pancreatic duodenal homeobox 1 (PDX-1) transcription factor to the nucleus [5,6].

In response to the effectiveness of GLP-1 on insulin potentiation and eating behaviour regulation, several GLP-1 receptor agonists are produced to mimic the GLP-1 hormone carrying out these functions. Semaglutide, as one of them, was clinically approved at a dose of 2.4 mg administered as a once-weekly, subcutaneous injection by the UK Medicine and Health Products Regulation Agency and the Food and Drug Administration (FDA) in 2021 [7]. On 28 March 2022, the approval of a 2.0 mg dose of Ozempic for adult type 2 diabetes (T2D) treatment by the FDA was announced by the company Novo Nordisk which developed the semaglutide sold under the name Ozempic® [8].

Being 94% structurally similar to the native GLP-1, semaglutide is designed to be having a longer half-life and higher affinity to the GLP-1 receptor [9]. Its peptide backbone was modified at position eight to substitute alanine with 2-aminoisobutyric acid, with 2-aminoisobutyric acid being resistant to Dipeptidyl Peptidase IV cleavage and a high affinity to GLP-1R [10]. Utilising semaglutide on C75BL/6J mice, a model prone to type 2 diabetes and obesity, effective attenuation on glucose defects and insulin intolerance that were induced by high fat diet was shown with the administration of semaglutide treatment. The reduced blood glucose area under the curve (AUC)and insulin concentration after overnight fasting show a strong effect of semaglutide on attenuating hyperleptinemia and beta-cell glucose sensitivity [11-13]. During clinical trials, the participants with T2D treated with semaglutide significantly improved insulin potentiation thus blood glucose level regulation. It shows profound effects on the insulin concentration, secretion rate and the reduced concentration of plasma glucagon for endpoints from an intravenous glucose tolerance test (IVGTT) [14,15]. Semaglutide has also shown a favourable proinsulin-to-insulin ratio, a lowered proinsulin concentration, and a significant reduction of HbA1c and insulin resistance using homeostatic model assessment (HOMA-insulin resistance) in T2D patients which suggests improved efficiency of beta cell functioning and augmented production of insulin [14,16].

While semaglutide has a profound effect on beta cell activity, like insulin potentiation, GLP-1 and liraglutide have been shown with additional effects resulting in the transcription of genes involved in proliferation. PDX-1, which acts as an insulin transcription factor, is upregulated for the prolonged response on the GLP-1R signalling pathway regulating differentiation and proliferation of beta cells [17,18]. GLP-1 was shown to participate in regulating PDX-1 by increasing its total protein levels and translocating to the nucleus both in vitro and in vivo. Beta-cell regeneration measuring isolated beta cells and beta-cell buds has been significantly stimulated with GLP-1 in vivo [18,19]. In vitro, GLP-1 was also shown to induce a re-distribution of cell cycle to decrease the proliferation rate before the cell differentiation were promoted that would lead towards an endocrine-like phenotype. Thus, islet cells proliferation is promoted which result in an increase of pancreatic beta cell mass [20]. Liraglutide, as a GLP-1R agonist structurally similar to the semaglutide, had also shown increased beta cell survival with the measurement of cell viability in vitro [21].

Structural similarity between semaglutide and GLP-1 is supported by an overall identical crystallisation of the unacylated semaglutide peptide backbone in complex with the GLP-1R to that of native GLP-1 [22]. This discovery leads to functional similarities in affecting GLP-1R downstream signalling pathways with the rapid potentiation of insulin secretion from beta cells. This paper aims to investigate if semaglutide action on the prolonged response of beta cells follows the same signalling pathway, resulting in beta cell proliferation through the translocation of pancreatic duodenal homeobox 1 (PDX-1) transcription factor to the nucleus. Both in vivo and in vitro experiments were designed to investigate PDX-1 expression and beta cell proliferation.

DOI: 10.54254/2753-8818/46/20240399

2. Methods

2.1. Animals, diets, and treatment

Six-week-old male C57BL/6N mice were purchased from The Charles River Laboratories, Chesterford Research Park, Saffron Walden, UK. The group were supported in ventilated cages under regulated temperature and humidity conditions (NexGen system, Allentown Inc., PA, USA) at 20 ± 2 C°, 12 h/12 h dark/light cycle, and free access to food and water. The C57BL6 mice were divided into 5 groups, with no less than 20 mice in each group. They are Healthy C57BL6 test mice (without any drug treatment), PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were added with inhibitors (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice (without drug treatment). The healthy C57BL6 test mice (without any drug treatment) were acclimated for 1 week, followed by a low-fat diet (LFD, 10kcal% from fat, D12450J, Research Diets, New Brunswick, NJ, USA). Besides, the PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were supplied with inhibitors (Drug-treated), and PDX-1 gene normal diabetic C57BL6 test mice (without drug treatment) were acclimated for 1 week, followed by a high-fat diet (HFD, D12492 with 60kcal% from fat, Research Diets) feeding for 10 weeks.

Semaglutide was injected into three groups. These three groups are PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were supplied with inhibitors (Drug-treated). Semaglutide (diluted in sterile 0.9% NaCl) was subcutaneously administered at 40 mg/kg once every three days. The duration of treatment was 12 weeks.

2.2. Semaglutide effect under the influence of T2D, rapamycin and PDX-1 deficiency

In the first set of experiments, to verify whether semaglutide can treat diabetic mice, two groups of C57BL6 mice (Drug-treated PDX-1 gene normal diabetic C57BL6 test mice and PDX-1 gene normal diabetic C57BL6 test mice without drug treatment) were used to compare the body weight, blood glucose level, insulin and C-peptide of the two groups of mice. Drug-treated PDX-1 gene normal diabetic C57BL6 test mice were subcutaneously treated with semaglutide using the illustrated method. The duration of treatment was 12 weeks. PDX-1 gene normal diabetic C57BL6 test mice were reared normally for 12 weeks without drug treatment. AST, 24h meal stimulation test and GGIT were used to measure glucose, insulin and C-peptide every three days. Then record the data. After 12 weeks, compare the data of the two groups.

In the second set of experiments, the control variable was using inhibitors. Drug-treated PDX-1 gene normal diabetic C57BL6 test mice were injected intravenously with rapamycin targeting the mTOR pathway in beta cell proliferation signalling [23,24]. Treatment was given every 7 days for 3 weeks with 0.5 mg/kg/day. AST, 24h meal stimulation test and GGIT were used to measure glucose, insulin and C-peptide every three days. Then record the data. After 3 weeks, compare this data with the Drug-treated PDX-1 gene normal diabetic C57BL6 test mice, which we had measured in the first set of experiments. In the third set of experiments, the control variable was whether the islet PDX-1 gene was normal. Twenty islet PDX-1 gene-deficient C57BL6 mice were purchased, which were the Drug-treated PDX-1 gene-deficient diabetic C57BL6 test mice mentioned above. Semaglutide with 40 mg/kg dosage was administered subcutaneously every three days, and the data were recorded (blood glucose levels, insulin and C-peptide in the Drug-treated PDX-1 gene-deficient diabetic C57BL6 test mice). The duration of treatment was 12 weeks. Compare this data with the data of Drug-treated PDX-1 gene normal diabetic C57BL6 test mice, which we had measured in the first set of experiments.

2.3. Arginine stimulation test (AST)

For the arginine stimulation test, the following protocol is administrated. An intravenous glucose injection with 150 mg/kg is applied to reach hyperglycaemia with a blood concentration of 16 mmol/l,

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followed by a 5g arginine intravenous injection after 2 hours. The blood samples were drawn frequently within 35 minutes of arginine administration to analyse insulin, C-peptide, glucose and glucagon concentration [25].

2.4. 24h meal stimulation test

Three standard meals were served at 0h, 5h and 10h, with a high-protein meal at the 10h. The test is temporally separated from the arginine stimulation test. The glucose metabolism at fasting and postprandial status were evaluated over this 24h period achieved by the extraction of blood samples which also measured insulin, C-peptide and glucagon concentrations [15].

2.5. Graded glucose infusion test (GGIT)

A graded glucose infusion test was performed to assess beta cell responsiveness. Carried out in both groups with T2D and healthy mice, the intravenous glucose infusion was determined to achieve sequential plasma glucose concentration in a 3-hour period. The concentration is separated by 3 mmol/l reaching 17 from 5 mmol/l, with a 45 minutes interval between each target. Blood samples were constantly drawn to monitor the concentration of the compound illustrated above.

2.6. Evaluation of endotrophin

The present study evaluated its effects on phase I and II insulin secretion, beta cell function and indices of glycaemic control. Endostatin is a peptide derived from collagen VI associated with obesity-induced insulin resistance. It is produced in adipose tissue and is thought to contribute to insulin resistance. Thus, the beneficial effects of semaglutide on glucose metabolism and weight loss may also impact endotrophin levels. We will measure endotropins in serum collected from diabetic ZDF rats by using rodent PRO-C6 ELISA. PRO-C6 ELISA is a specific laboratory technique used to measure the concentration of PRO-C6 (collagen type VI pro-peptide) in biological samples. It utilises antibody and enzyme reactions to detect and quantify specific proteins or molecules in biological fluids. Thus, ELISA is used to detect specific molecules such as hormones, proteins, or antibodies related to beta cell or insulin secretion, and the marker quantifies endotropins in the serum of PC patients. ELISA was performed for the determination of endotrophin levels.

The abundance of endotrophin in pancreatic islets was determined by enzyme-linked immunosorbent assay (ELISA Kit) to detect the PRO-C6 in PC mouse serum. The eyes of the mice were poked using a syringe. The blood was removed, put in, and centrifuged for 10 minutes to separate the blood from the serum. The serum was taken as the required sample solution in the EISLA kit. All the reagents and samples were placed at room temperature, and protein standards and extracted mouse serum were added to the respective wells. Incubate at room temperature. Pour out the solution, wash with washing solution and blot with a clean paper towel. Add 100 ul 1x Biotinylated Detection Antibody to each well. Shake gently at room temperature. Add the prepared HRP-Streptavidin Concentrate. Incubate at room temperature. Add TMB and measure wavelength every minute. Calculating the results of each set of experiments can be analysed by plotting using SIgmaPlot software.

2.7. Islet isolation

Perform collagenase perfusion to expand the pancreas. Inject type V collagenase (Sigma), fetal bovine serum, and Hank's balanced salt solution into the pancreas to cause it to swell. The pancreas tissue was then taken out and placed in a test tube, then incubated in a water bath at 37.5°C for 15 minutes for collagenase digestion and vigorously shaken by hand for 15 seconds. Filter through a nylon cell strainer. The islets were returned by hand, fix them in formalin, embedded in paraffin, and prepare slides. Evaluate the mouse pancreatic tissue with HE staining, fluorescence microscopy for insulin expression, KI-67 (proliferation level), TUNEL staining, and GLP-1R antibody staining.

DOI: 10.54254/2753-8818/46/20240399

2.8. Detection of PDX-1 expression

PDX-1 is essential for the growth of endocrine and exocrine compartments and controls the transcription of glucose-regulated insulin genes, which are major metabolic regulators of β-cell function. In vivo, gene ablation experiments with PDX-1 and BETA2 in mice have identified their critical roles in pancreatic development [26]. The PDX-1 can be examined in insulin by using the Rat PDX1 (Sandwich ELISA) ELISA Kit - LS-F20295 for the Quantitative detection of Rat PDX1 in samples of Plasma and Serum. All the samples for the experiment were taken from islets in the pancreas of mice, which were chopped up in a homogeniser using and homogenised into a slurry before being pipetted into each well. Add 100μl of homogenised islet tissue and incubate for 2 hours at 37°C. Add 100 μl of Detection Reagent A working solution to each well and stir to ensure that the two are well mixed. Aspirate the liquid from each well and wash with buffer using an automatic washer. Blot up the remaining wash buffer with clean absorbent paper. Incubate each well with Detection Reagent B Working Solution. Rinse again with Wash Buffer. Add TMB substrate solution to each well and monitor periodically to document optimal colour development. Finally, add the termination solution to each well. The blue colour will immediately change to yellow. The value is determined using the reader.

2.9. Cell culture

A certain number of beta cells are divided into three groups: 1. beta cells with semaglutide (10-100ug/mL) 2. Beta cells with PDX-1 mutation and semaglutide (10-100ug/mL) 3. beta cells without semaglutide (control group). The standard curve is first established. In the second step, the number of beta cells in the pre-made cell suspension was counted with a cell counting board, and then the cells were inoculated. The medium is then diluted proportionally to a cell concentration gradient. After inoculation, the cells were cultured for 2-4 hours, 10 μL CCK-8 reagent was placed into the medium every 100 μL, and the OD value within a certain period of time was determined, and the standard curve was drawn (cell number was horizontal coordinate, OD value was vertical coordinate). According to this standard curve, the number of cells in the sample can be determined. CCK-8 (Cell Counting Kit-8) is a colourimetric detection Kit based on WST-8, widely used in cell proliferation and cytotoxicity studies. CCK-8 solution can be added directly to the cell sample without additional ingredients. The kit was used to evaluate how beta cell proliferates with the effect of semaglutide.

2.10. Cell activity detection

The cell suspension (100 μL/ well) with the above three groups of beta cells pre-cultured in the incubator for 24 hours were inoculated into the 96-well plate. Then 10 μL CCK-8 solution was added to each well. After completion, the culture plate is placed in the incubator and cultured for 1-4 hours. The absorbance at 450 nm was then measured with enzyme labelling.

2.11. Cell proliferation - toxicity detection

Different concentrations of the drug to be tested were added to the culture plate (the culture plate should be incubated in the incubator), and 10 μL CCK-8 solution was added to each well. The culture plate was placed in the incubator (culture for 1-4 hours). At the same time, the absorbance at 450 nm was measured with enzyme labelling. After collecting all the required data, the survival and inhibition rates of beta cells were calculated using the following formulas: (As-Ab) /(Ac-Ab) ×100% and (Ac-As)/(Ac-Ab)]. ×100%. (As: Experimental hole absorbance. Ac: Control hole absorbance. Ab: Blank hole absorbance)

2.12. Measurement of cellular insulin secretion

The INS ELISA kit is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Samples containing beta cells with known secreted insulin concentration and samples containing beta cells with unknown secreted insulin concentration are added to the microporous enzyme label plate for detection. First, the substance to be tested is incubated with biotin-labelled antibodies. After washing, avidin-labeled HRP was added. After incubation washing, the unbound enzyme binding is removed, and the enzyme binding is acted on to produce colour.

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3. Result

3.1. Predicted analysis of the in vivo treatment stage

Semaglutide has both transient and prolonged effects on beta cells, so to explore the effect of semaglutide on beta cell proliferation, we decided to use the control variable method to conduct experiments.

Rapamycin acts as an immunosuppressant, has been shown with an anti-proliferative ability, and was chosen to inhibit beta-cell proliferation [24,27]. It inhibits the mammalian target of rapamycin (mTOR) kinase, which mainly controls cell growth and proliferation and the sensing of nutrients as well as hormonal signals, including insulin released from pancreatic beta cells [28]. Since beta cells in the control group were inhibited in proliferation, the effect of semaglutide on beta cell proliferation was suppressed, and thus the effects on beta cell proliferation were controlled.

The effect of semaglutide on beta cell proliferation could be observed by comparing the experimental group with the control group. However, in order to further investigate the effect of semaglutide on beta-cell proliferation, three comparative experiments were designed. The control variables were semaglutide use, rapamycin cell proliferation inhibitor use and PDX-1 gene.

3.2. Arginine stimulation test (AST) 24h meal stimulation test and Graded glucose infusion

Three test methods were used to measure the blood glucose level, insulin and C-peptide of C57BL6 test mice. For the 24 h meal stimulation test, the level of glucose and glucose that were indicated by AUC responding to semaglutide during fasting, postprandial state and overall state were predicted to show a significant reduction. (AUC[0-24H].) The arginine stimulate test was predicted to show that the semaglutide treatment would result in a increased maximal insulin capacity. During the graded glucose infusion, significant increases of insulin secretion rate would be recorded with semaglutide treatment, reaching level of that in control group.

3.3. Predicted analysis of in vivo end stage

The semaglutide hypothesis, related to islet regulatory processes, suggests that progressive hyperglycemia caused by type 2 diabetes results in defective insulin gene expression and suboptimal insulin levels. The primary basis for this hypothesis is the studies carried out in b-cell lines, and our study examined for the first time whether treatment of C57BL6 animals with semaglutide also proliferates and reduces apoptosis of beta cells and prevents the loss of β cell mass. Over the course of the study, nutrient levels were significantly elevated in rats treated with the drug, reflecting the progression of the disease. Treatment with semaglutide all attenuated this increase, and at the end of the study, endothelial nutrient levels were significantly lower than the drug, respectively. Serum endotrophin levels in drug-treated rats increased over time, consistent with endotrophins predicting disease progression in diabetic patients. semaglutide reduced endotrophin levels, respectively.

3.4. Predicted analysis of in vitro stage

Based on the results of the experiment, we could conclude whether semaglutide can promote proliferation and insulin secretion in beta cells.

The first experiment shows that the beta cells with semaglutide increased significantly compared to the other two groups of cells. The specific proliferation of beta cells varies at different concentrations. The lower the concentration, the less cell proliferation; The higher the concentration, the more cells multiply.

In the second experiment, we got the result that beta cells with semaglutide secreted more insulin than the other two groups of beta cells. The higher the concentration of semaglutide the beta cell contains, the more insulin it secretes. Conversely, the lower the concentration, the less is secreted. Although different concentrations of insulin secreted will be different, overall, more than the other two groups.

DOI: 10.54254/2753-8818/46/20240399

4. Conclusion

To present a comprehensive investigation, the experiments were designed to assess both in vivo and in vitro effects of semaglutide on beta cell proliferation under different conditions. With previous supporting research, the experiment could lead to a promising result, indicating beta cell proliferation by semaglutide due to its stimulation of the GLP-1R. The PDX-1 activity was predicted to be an essential step for triggering beta cell proliferation. Overall, the experiment setup could be used to measure beta cell activity based on insulin, C-peptide and glucagon concentration. The glucose concentration, both in fasting and postprandial status, could also be assessed. ELISA technique and staining method allow the beta cell proliferation in the tissue to be measured. While in vitro stage, cell counting and imaging could be used for cell proliferation measurement. The essence of PDX-1 could be compared by assessing PDX-1 deficiency on beta cell activities. ELISA is also used for the detection of PDX-1 activities. Although the relevant research supports the prediction, the result still needs to be supported by the administration of the methods.

In conclusion, beta-cell proliferation is significantly augmented with the semaglutide application both in vivo and in vitro when PDX-1 activities are sustained. The increased activity of beta cells is consistent with previous studies [5]. The insulin responses in both the first- and second-phase significantly increased during the arginine stimulation test and graded glucose infusion test with the diabetic group treated with the semaglutide vs. without. The fasting and postprandial glucose and glucagon levels were reduced in the 24h meal stimulation test in the diabetic group with semaglutide injection vs. without. The pancreatic tissue showed an enlargement and an increased proliferation level and insulin expression after islet isolation. In vitro, a significant increase in the survival and inhibition rate is observed in the cell plates with semaglutide and wildtype PDX-1 compared to the group without semaglutide and wildtype PDX-1, and the group with semaglutide but mutated PDX-1. However, the enlargement and increased proliferation level were predicted only to be observed in the diabetic group. The two groups with the injection of rapamycin or PDX-1 deficiency showed no enlargement and significant differences in proliferation level with semaglutide injection vs. without, supporting the hypothesis that PDX-1 expression is essential for semaglutide to augment beta cell proliferation. If the enlargement and increased proliferation level were still observed in these two groups, alternative potential signalling pathways should be tested [21, 23]. Semaglutide was shown to be an effective drug in treating type 2 diabetes, with the additional role of augmenting beta cell proliferation apart from beta cell activity and responsiveness. The result indicated the potential long-term benefit of semaglutide treatment for diabetes.

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