Original Paper

Pharmacological Effects of EGLP-1, a Novel Analog of Glucagon-Like Peptide-1, on Carbohydrate and Lipid Metabolism

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Key Words

EGLP-1 • NEFA • STZ-induced hyperglycemia mice • PHSL

Abstract

Background/Aims: Abnormal glucose metabolism and lipid metabolism are two key issues in Type 1 diabetes mellitus (T1DM). Insulin can control carbohydrate metabolism adequately, but cannot regulate lipid metabolism well in patients with T1DM. Glucagon-like peptide-1 receptor agonists (GLP-1RAs) have cured type 2 diabetes mellitus in clinical trials and have improved T1DM glycemic control in preclinical studies. However, previous studies have not reported whether GLP-1 can lower the serum concentration of non-esterified fatty acids (NEFAs). In this study, we examine whether GLP-1 can affect serum NEFA levels. **Methods:** The bioactivity of EGLP-1 (a novel GLP-1 analog) *in vitro* was analyzed in CG-HEK293 cells and with high-performance liquid chromatography. An intraperitoneal glucose tolerance test (IPGTT) was used to analyze the acute and sustained hypoglycemic effects of EGLP-1 in normal C57BL/6J mice. Streptozotocin-induced hyperglycemic mice were used to study the effects of EGLP-1 on blood glucose and NEFAs as well as its mechanism. **Results:** EGLP-1 activated GLP-1R and resisted dipeptidyl peptidase-IV digestion *in vitro*. Additionally, EGLP-1 had an insulinotropic action *in vivo* that lasted for approximately 6 h. In Streptozotocin-induced hyperglycemic mice, EGLP-1 improved hyperglycemia, inhibited food intake, and increased β -cell area. Serum physiological indexes showed that insulin and C-peptide levels were increased, while NEFA and triacylglycerol concentrations were decreased. Western blot analysis revealed that EGLP-1 significantly reduced phosphorylated-hormone sensitive lipase (pHSL) levels in white adipose tissue. **Conclusions:** EGLP-1 can improve hyperglycemia by increasing islet β -cell area and improving β -cell function, possibly due to reduced NEFA content in serum by lowering pHSL levels.

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Introduction

Type 1 diabetes mellitus (T1DM) is caused by an absolute lack of insulin due to autoimmune destruction of pancreatic β -cells [1]. Over the past few years, considerable research has been done on both disease prevention and treatment [2, 3]. Although great progress has been made in the management of T1DM, the mainstay of clinical treatment remains insulin injections. Insulin is a potent regulator of blood glucose and enables patients to avoid hyperglycemia and its sequelae. However, patients with T1DM also have altered metabolism that favors lipid oxidation over carbohydrate oxidation. Compared with healthy individuals, patients with T1DM have both increased lipid oxidation and increased serum levels of non-esterified fatty acids (NEFAs) [4-7]. Insulin, however, is not a powerful regulator of lipid metabolism and cannot prevent such complications.

The incretin hormone glucagon like peptide-1 (GLP-1) is released from intestinal L-cells into the circulation following the ingestion of nutrients [8, 9]. GLP-1 promotes insulin secretion, inhibits glucagon release, delays gastric emptying, and minimizes postprandial glucose excursions [10-12]. GLP-1 receptor agonists (GLP-1RAs), such as exenatide and liraglutide, have been used clinically to treat type 2 diabetes mellitus (T2DM) with a more notable improvement of controlling blood glucose than insulin alone therapy. More recently, GLP-1RAs have been included into the treatment regimen for T1DM, with benefits including a greater reduction in hemoglobin A1c, insulin dose, and risk of hypoglycemia [13]. In animal model studies, in addition to stimulating glucose-dependent insulin secretion, GLP-1 inhibits hepatic glucose production, protects the heart as an antioxidant, relieves oxidative stress in the vascular system, and affects bone metabolism [14-18].

In summary, GLP-1RAs are potential drugs for the treatment of T1DM, but it has not been reported whether GLP-1 can decrease the serum concentration of NEFAs. In this study, we suggest a mechanism through which GLP-1 affects serum NEFA content. However, due to the short half-life (2-3 min) of natural GLP-1 [19], it is difficult to study its role as a receptor agonist directly. Therefore, our laboratory previously designed a peptide GLP-1 analog, called EGLP-1, which can resist degradation by dipeptidyl peptidase-IV (DPP-IV), and we studied its hypoglycemic action as well as its effect on NEFA lipid metabolism.

Materials and Methods

Peptide synthesis

GLP-1, exendin-4, and EGLP-1 were chemically synthesized by GenScript (Nanjing, China).

Biological activities in vitro

The CG-HEK293 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing (25 mM glucose, 10% fetal bovine serum (FBS), 100 μ g/mL penicillin, 100 μ g/mL streptomycin, 50 μ g/mL G418, and 0.5 μ g/mL puromycin), and was used to test GLP-1R activity. GLP-1R activation by EGLP-1 was analyzed *in vitro* as described previously [17].

In vitro, a stability test of EGLP-1 against DPP-IV enzymolysis was carried out as described previously [17]. Briefly, GLP-1 or EGLP-1 was mixed with an isopycnic volume of recombinant human DPP-IV enzyme (R&D Systems, Minneapolis, MN) and incubated for different times at 37 °C. A reversed phase high-performance liquid chromatography (HPLC) system was used to analyze the remaining concentration of GLP-1 or EGLP-1 in the samples.

Culturing of 3T3-L1 adipocytes

Mouse 3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS, 100 μ g/mL penicillin and 100 μ g/mL streptomycin at 37°C and 5% CO₂ in a humidified incubator. A mature adipocyte induction program was used: 1) 3T3-L1 pre-adipocytes were cultured in normal medium until confluency; 2) at 2 days post-confluency (day 0), the cells were stimulated with DMEM induction media containing 10% FBS, 5 μ M dexamethasone, 0.5

µg/mL insulin, and 0.5 mM isobutylmethylxanthine; 3) after 2 days (day 2), the medium was changed to differentiation medium II containing DMEM supplemented with 10% FBS and 0.5 µg/mL insulin; and 4) at 2 days later (day 4), the medium was changed to normal medium, and the cells were fed with 10% FBS/DMEM every 2 days until day 10. Mature 3T3-L1 adipocytes were treated with phosphate-buffered saline, EGLP-1 (10 µM), exendin-4 (10 µM), or GLP-1 (10 µM) for 12 h for the following experiment.

Animals

C57BL/6J mice (7-8 weeks old) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). The mice were housed in the animal facility on a 12 h light-dark cycle at the Pharmaceutical Animal Experiment Center. All animals were fed according to the protocols of the National Institutes of Health and permission was granted by the China Pharmaceutical University Institute Animal Care and Use Committee (Nanjing, China).

An intraperitoneal glucose tolerance test (IPGTT) was performed with 1.5 g/kg glucose in C57BL/6J mice (8-10 weeks old). To detect the effective time of EGLP-1 *in vivo*, the mice were injected subcutaneously with placebo, GLP-1 (30 nmol/kg), exendin-4 (30 nmol/kg) or EGLP-1 (30 nmol/kg). The IPGTT was performed at set time points after injection.

The mice were injected intraperitoneally with streptozotocin (STZ; Sigma, St. Louis, MO; 50 mg/kg body weight) for 5 consecutive days [20, 21]. Fasting blood glucose was measured with a blood glucose meter (OMRON, Kyoto, Japan). Mice with 2 consecutive blood glucose measurements greater than 11.1 mM after the last injection of STZ were considered hyperglycemic.

Hyperglycemic model mice were divided into 4 groups as Vehicle, GLP-1, exendin-4, and EGLP-1. All groups were injected subcutaneously twice daily with placebo or 30 nmol/kg of peptides, respectively, for 5 weeks. Body weight and fasting blood glucose (fasted for 8 h) were measured once a week. At the end of the dosing cycle, blood samples were collected via retro-orbital bleeding. The levels of serum NEFAs and triglycerides (TGs) were determined by specific enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Institute of Research, Nanjing, China). The levels of serum insulin, glucose, adiponectin, and C-peptide were determined by ELISA kits (Nanjing Jian Cheng Institute, Nanjing, China).

Pancreas morphometry

The pancreas was processed and stained with hematoxylin and eosin. Sections were deparaffinized, rehydrated, and immunostained for insulin (dilution 1: 300; Abcam, Cambridge, UK). The sections were examined, scanned, and analyzed as described previously [22]. The β-cell area per whole pancreas was then calculated.

Real-time PCR

Mouse subcutaneous white adipose tissue from the thighs and buttocks was pulverized in liquid nitrogen and total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared by HiScript Q RT SuperMix for qPCR (Vazyme, Nanjing, China), and real-time PCR assays were carried out with an LC480 Light Cycler (Roche, Mannheim, Germany) using specific primers for hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL).

Western blot analysis

Subcutaneous white adipose tissue from the thighs and buttocks was lysed, boiled and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked with Western Blot Blocking Solution (Beyotime, Shanghai, China). The following primary antibodies were used: anti-phospho-HSL (Ser563) (Cell Signaling, Danvers, MA), anti-HSL (Santa, Cruz Biotechnology, Dallas, TX), and anti-GAPDH (Santa, Cruz Biotechnology, Dallas, TX). To quantify optical density, TanonImage 1.00 software (Tanon Science & Technology, Shanghai, China) was used.

Statistical analysis

All data are expressed as the means ± standard error of the mean and were analyzed using Student's t-test or one-way analysis of variance followed by the Student-Newman-Keuls *post hoc* test by SPSS software (SPSS Inc., Chicago, IL).

Results

Sequence design and bioactivity analysis of EGLP-1 *in vitro*

In order to prolong the half-life of GLP-1, EGLP-1 was designed as follows: the Ala8 was replaced by Gly, and the sequence of SSGAPPPS obtained from exendin-4 was linked to the end of GLP-1 (Fig. 1a).

Subsequently, EGLP-1 was analyzed for receptor-ligand binding activity *in vitro*. As shown in Fig. 1b, EGLP-1 was able to activate GLP-1R in a dose-dependent manner. The EC₅₀ of EGLP-1 was 10.46 nmol/L, which was not significantly different from the positive control GLP-1.

In vitro, the stability of EGLP-1 against DPP-IV enzymolysis was evaluated by HPLC [23]. GLP-1 or EGLP-1 was incubated with DPP-IV at 37°C for 1–10 h and the proportion of remaining intact peptides was analyzed. The results showed that GLP-1 was degraded rapidly. After incubation for 1 h, only approximately 40% of the complete peptide remained, and after 10 h, only 6.3% of intact GLP-1 remained. However, EGLP-1 was able to resist of DPP-IV enzymolysis completely, and after 10 h of incubation, almost 100% of EGLP-1 remained intact (Fig. 1c).

Acute and sustained hypoglycemic effect of EGLP-1 *in vivo*

To investigate the dose-response relationship of EGLP-1, an IPGTT was performed following subcutaneous injection of native GLP-1 (30 nmol/kg), exendin-4 (30 nmol/kg), or EGLP-1 (0.1, 1, 30, or 100 nmol/kg). The results revealed that blood glucose was significantly lowered in all peptide-treated groups and there was no significant difference between the groups under the same dose. Furthermore, the minimum dose of EGLP-1 for a hypoglycemic effect was 0.1 nmol/kg (Fig. 2a). These results verified that EGLP-1 had acute insulinotropic activity and the minimum effective concentration was 0.1 nmol/kg.

To evaluate the effective time of EGLP-1 *in vivo*, an IPGTT was performed at 0, 1, 2, 4, 6, or 8 h after the subcutaneous administration of a single dose (30 nmol/kg) of EGLP-1, GLP-1 or exendin-4 in normal C57BL/6J mice. As shown in Fig. 2b, the hypoglycemic effect of EGLP-1 was still evident at 4 h after administration, and this trend continued for up to 6 h. In contrast, the hypoglycemic effect of native GLP-1 lasted for less than 1 h, confirming that EGLP-1 could work as a GLP-1R agonist, although exendin-4 had a longer glucose-lowering action of approximately 6 h.

Fig. 1. Design and biological activity of EGLP-1. (a) Sequences of EGLP-1, GLP-1, and exendin-4. (b) Receptor-ligand binding activity of GLP-1 or EGLP-1 in HEK293/GLP-1R/GFP cells. (c) Stability of EGLP-1 against DPP-IV degradation *in vitro*.

Fig. 2. Acute dose-dependent glucose-lowering activity of EGLP-1 in fasted C57BL/6J mice. (a) An IPGTT was performed after the intraperitoneal administration of placebo, GLP-1, exendin-4, or EGLP-1. (b) Blood glucose levels in fasted C57BL/6J mice during an IPGTT performed at different times after the subcutaneous administration of GLP-1, exendin-4 or EGLP-1. * $p < 0.05$ and *** $p < 0.001$, EGLP-1 group compared with the Vehicle group; ^^^ $p < 0.001$, GLP-1 group compared with the Vehicle group; ### $p < 0.001$, Exendin-4 group compared with the Vehicle group; \$\$\$ $p < 0.001$ EGLP-1 group compared with the GLP-1 group.

Fig. 3. Chronic effect of GLP-1, EGPL-1, and exendin-4 on blood glucose, Δ body weight, cumulative food intake, and pancreas in hyperglycemic C57BL/6J mice. Weekly fasted blood glucose level (a), cumulative food intake (b), and body weight (c) during subcutaneous injection of GLP-1 (30 nmol/kg), EGPL-1 (30 nmol/kg), exendin-4 (30 nmol/kg) or vehicle twice daily for 5 weeks. Hematoxylin-eosin staining of the pancreas (d) of every group after 5-week treatment. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, EGPL-1 group compared with the Vehicle group; ## $p < 0.01$, and ### $p < 0.001$, Exendin-4 group compared with the Vehicle group; \$\$\$ $p < 0.01$, and \$\$\$\$ $p < 0.001$, EGPL-1 group compared with the GLP-1 group; & $p < 0.05$ and &&& $p < 0.001$, EGPL-1 group compared with the Exendin-4 group.

Chronic activity of EGPL-1 in STZ-induced hyperglycemic mice

Following the administration of EGPL-1 to STZ-induced hyperglycemic mice, their weekly fasting blood glucose began to show a decline. After 4 weeks of EGPL-1 treatment, their fasting blood glucose was significantly different compared with placebo. At week 5, EGPL-1-treated mice had a fasting blood glucose level of only 8.31 mmol/L (compare with the Vehicle group, $p < 0.01$). As for the positive controls, that is, the GLP-1 and Exendin-4 groups, both demonstrated only a little decrease (GLP-1 group = 12.2 mmol/L, Exendin-4 group = 13.3 mmol/L) in fasting blood glucose, with no significant difference compared with the Vehicle group (Fig. 3a). Additionally, treatment with EGPL-1 reduced food intake. After 14 days, the p value was less than 0.001 when comparing EGPL-1 treated mice with the Vehicle or GLP-1 treated group. During the 21 days of this experiment, cumulative food intake for EGPL-1 mice was 59.9 g, and there was a significant difference between the EGPL-1 and Exendin-4 groups (Fig. 3b). Although EGPL-1 had a significant effect on food intake, it had no significant effect on body weight (Fig. 3c).

EGLP-1 treatment increases β -cell area

Hyperglycemia induced by STZ treatment is mainly caused by chemical toxicity to β -cells in pancreatic islets. GLP-1R agonists can protect pancreatic endocrine β -cells, and pancreatic islet integrity was analyzed via hematoxylin and eosin staining. The pancreatic islets of the Vehicle group were seriously damaged (Fig. 3d). However, in the EGPL-1, GLP-1, and Exendin-4 groups, pancreatic islet damage was significantly alleviated, and the islet structures appeared more intact (Fig. 3d). In order to verify whether there were larger numbers of islet β -cells, insulin immunofluorescence staining of the pancreas was performed. The pancreatic islets β -cells area of the Vehicle group was significantly decreased compared to the EGPL-1, GLP-1, and Exendin-4 groups (Fig. 4a). Islet β -cells area analysis showed that islet area was increased by 2-fold in the GLP-1, Exendin-4, and EGPL-1 groups compared

Fig. 4. Pancreatic immunoassay in hyperglycemic C57BL/6J mice after 5-week treatment. (a) Pancreatic β -cell immunostaining; (b) analysis of β -cell area in pancreas (10/10); and (c) analysis of islet number in the pancreas.

with the Vehicle group (Fig. 4b). There was no significant difference in the total number of islets between the groups (Fig. 4c).

Although pancreatic endocrine β -cells play an important role in blood glucose control, β -cell dysfunction is common in diabetic patients independent of the method of treatment [24]. Therefore, we assessed the concentrations of serum insulin and C-peptide, which were increased significantly after EGLP-1 treatment (compared with the Vehicle group, $p < 0.05$) (Fig. 5a, b).

EGLP-1 improves physiological indexes

Diabetic patients are known to have not only abnormal glucose metabolism, but also abnormal lipid metabolism. Thus, we observed serum TG and NEFA levels in our STZ-induced hyperglycemic mice. EGLP-1 treatment significantly decreased TG and NEFA levels (TGs: EGLP-1 vs. Vehicle, $p < 0.001$; NEFAs: EGLP-1 vs. Vehicle, $p < 0.01$) (Fig. 5c, d). Serum glucagon was also decreased, but not significantly (compared with the Vehicle group, $p = 0.1535$) (Fig. 5e). Adiponectin also did not decline significantly (Fig. 5f).

EGLP-1 reduces serum NEFA level by decreasing phosphorylated-HSL (pHSL) protein levels in adipose tissue

Adipose tissue lipolysis, as a simple and well-understood metabolic pathway, is an important source of NEFAs. During lipolysis, intracellular triacylglycerol is broken down into NEFAs and glycerol through the action of three major lipases: ATGL, HSL and MGL [25, 26]. Compared with the Vehicle group, the mRNA levels of ATGL, HSL, and MGL in adipose tissue were not changed in mice treated with EGLP-1, GLP-1, and exendin-4 (Fig. 6a-c). We examined the protein expression of key enzymes in adipose tissue to analyze the effect of EGLP-1 on lipolysis. The protein level of HSL in adipose tissue was not changed in mice treated with EGLP-1 compared to those treated with vehicle. However, pHSL, the main active form of HSL, decreased by approximately 5-fold in adipose tissue of mice receiving EGLP-1 (Fig. 6d, e).

Fig. 5. Physiological indexes in hyperglycemic C57BL/6J mice after 5-week treatment. Concentration of serum insulin (a), C-peptide (b), TGs (c), NEFAs (d), glucagon (e), and adiponectin (f) after 5-week treatment. ** $p < 0.01$, and *** $p < 0.001$, EGLP-1 group compared with the Vehicle group; ## $p < 0.01$, Exendin-4 group compared with the Vehicle group; \$ $p < 0.05$ and \$\$\$ $p < 0.001$, EGLP-1 group compared with the GLP-1 group.

Fig. 6. Analysis of key enzymes in adipose tissue lipolysis. Quantitative real-time PCR measurement of the mRNA levels ($n = 6$ mice per group) of (a) HSL, (b) ATGL, and (c) MGL. (d) Expression of HSL and pHSL, and (e) quantification of the western blots ($n = 3$ mice per group). *** $p < 0.001$, EGLP-1 group compared with the Vehicle group; ### $p < 0.001$, Exendin-4 compared with the Vehicle group; && $p < 0.001$, EGLP-1 group compared with the Exendin-4 group; ^^^ $p < 0.001$, GLP-1 group compared with the Vehicle group.

Fig. 7. Analysis of key enzymes in mature 3T3-L1 adipocytes. (a) Expression of HSL and pHSL, and (b) quantification of the western blots ($n = 3$). *** $p < 0.001$, EGLP-1 group compared with the Vehicle group; ^^ $p < 0.01$, GLP-1 group compared with the Vehicle group.

EGLP-1 reduces pHSL protein levels in mature 3T3-L1 adipocytes

Similar findings of decreased adipose tissue lipolysis were observed in mature 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were incubated with phosphate-buffered saline, exendin-4 (10 μ M), EGLP-1 (10 μ M), or GLP-1 (10 μ M) for 12 h. EGLP-1 and GLP-1 treatment significantly decreased the level of pHSL, but exendin-4 did not. EGLP-1, GLP-1 and exendin-4 did not change the protein level of HSL (Fig. 7a, b).

Discussion

GLP-1 exerts a large number of antidiabetic actions, of which the induction of glucose-dependent insulin secretion is best known [9]. Both GLP-1 agonists and inhibitors of the enzyme DPP-IV are approved for the treatment of T2DM [27, 28]. It has been reported that GLP-1 can improve T1DM by lowering the insulin dose, hemoglobin A1c, and risk of hypoglycemia [29-31]. Carbohydrate metabolism and lipid metabolic disorders are two key features of T1DM and T2DM. Especially in T1DM patients, lipid oxidation is greatly increased, and NEFAs are higher than in normal subjects [7]. In this report, our laboratory discovered that EGLP-1, as a long-acting analog of GLP-1, could lower hyperglycemia and reduce the concentrations of NEFAs and TGs in STZ-induced hyperglycemic mice.

To obtain a long-acting analog of GLP-1, the sequence of SSGAPPPS obtained from the tail of exendin-4 was linked to the end of GLP-1, which was designated as EGLP-1. *In vitro*, receptor-ligand binding activity showed that EGLP-1 could activate GLP-1R and had a similar affinity with GLP-1 (Fig. 1b). Consistent with receptor-ligand binding activity *in vitro*, similar to native GLP-1, EGLP-1 promoted insulin secretion *in vivo* with a minimum effective concentration of 0.1 nm/kg (Fig. 2a). *In vitro*, HPLC revealed that EGLP-1 almost completely resisted digestion by DPP-IV. When EGLP-1 was incubated with DPP-IV for up to 10 h, less than 10% of EGLP-1 was degraded. Comparatively, only 6.3% of GLP-1 remained intact after DPP-IV digestion (Fig. 1c). *In vivo*, an IPGTT revealed that a single injection of EGLP-1 could reduce blood glucose for about 6 h (Fig. 1c). This is consistent with the report of Finan et al., who also added the tail of exendin-4 to a peptide triagonist, and the resultant compound had a half-life of about 5 h in normal C57BL/6J mice [32]. However, the effective time of EGLP-1 was less than that of exendin-4, probably because the structure of GLP-1 is different from that of exendin-4. In the following study, we improved the half-life of EGLP-1 by changing its dosage *in vivo*.

To verify the effectiveness of EGLP-1 *in vivo*, we administered EGLP-1, GLP-1, exendin-4, or vehicle to STZ-treated hyperglycemic mice. After 5 weeks of treatment, EGLP-1 clearly ameliorated the hyperglycemia (compared with the Vehicle group, $p < 0.01$). However, neither GLP-1 nor exendin-4 demonstrated any improvement compared with vehicle (Fig. 3a). Consistent with the report that GLP-1R agonist was able to inhibit food intake through central regulation [33-35], EGLP-1 significantly inhibited food intake, and it even had a comparatively better effect than exendin-4 from the third week onward (Fig. 3b). This indicated that EGLP-1 can act as an effective GLP-1R agonist.

Both EGLP-1 and exendin-4 are long-lasting GLP-1R agonists, but they have different hyperglycemic effects. Why? It is well known that pancreatic endocrine β -cells play a central role in glucose metabolism, so we analyzed pancreata by hematoxylin and eosin

staining, which revealed that the pancreas of the Vehicle group was seriously damaged. In comparison, EGLP-1, GLP-1, and exendin-4 treatment preserved islet integrity. This indicated that these GLP-1R agonists had a protective effect on islet morphology, which is consistent with our previous report [17]. It has also been reported that GLP-1 can increase β -cell mass by stimulating β -cell proliferation and inhibiting apoptosis [36, 37]. Islet immunostaining for insulin showed that EGLP-1, GLP-1 and exendin-4 treatment significantly increased the β -cell area compared with the Vehicle group (Fig. 4), but there was no significant difference between EGLP-1, GLP-1 and exendin-4 treatment. In order to detect whether the β -cells were able to secrete insulin, we detected serum insulin and C-peptide levels. The results showed that EGLP-1 treatment significantly increased insulin and C-peptide levels (Fig. 5a, b). These results indicated that EGLP-1 reduced blood glucose by improving the function of β -cells.

It has been reported that GLP-1 possesses a number of anti-diabetic actions, including glucose-dependent insulin secretion (insulinotropic action) dependent on GLP-1R [9] and an insulin-like (insulinomimetic) action independent on GLP-1R [38-41] in addition to its insulinotropic action. This suggests that EGLP-1 may have a new role in improving β -cell function independently of GLP-1R. Exendin-4, a GLP-1R agonist, lacks insulinomimetic action, while GLP-1 has insulinomimetic action, but its half-life is only 2-3 min [19]. EGLP-1 is not only a long-acting GLP-1R agonist, but also retains the structure of GLP-1, so it may have possess of their abilities to increase C-peptide and insulin levels.

It has been reported that elevated levels of NEFAs contribute to the impaired responsiveness to GLP-1, partially through the down-regulation of GLP-1 receptor signaling. Improvements in lipid control in mouse models of obesity and diabetes increase the efficacy of incretin-based therapies [42]. It is recognized that NEFAs have stimulatory and detrimental effects on pancreatic β -cells. The acute exposure of pancreatic β -cells to saturated NEFAs results in a substantial increase in insulin release, whereas chronic exposure results in desensitization and suppressed secretion, which then induces apoptosis [43, 44]. In addition, the STZ-induced hyperglycemic mouse model has the features of dyslipidemia, increased β -oxidation, and elevated levels of TGs and NEFAs [7, 17, 45]. Therefore, we measured NEFA concentration in our experimental groups. The results showed that the concentration of NEFAs was significantly decreased in the EGLP-1 group (Fig. 5d), suggesting that EGLP-1 may improve β -cell function by decreasing serum NEFA levels. In order to further study the mechanism by which EGLP-1 reduces NEFAs, we detected the expression of HSL, a key enzyme of lipid metabolism, and pHSL, which is the main active form of HSL [25]. Western blotting analysis showed that EGLP-1 significantly reduced pHSL levels (Fig. 6). In studies of mature 3T3-L1 adipocytes, we obtained similar findings in that EGLP-1 significantly decreased pHSL levels. This indicated that EGLP-1 reduced serum NEFA levels by reducing pHSL levels, which may improve the function of β -cells.

Overall, the results of this study suggest that EGLP-1 can reduce hyperglycemia and lower the concentration of serum NEFAs. *In vivo and in vitro*, biological activity analyses confirmed that EGLP-1 is a long-acting GLP-1R agonist that exerts an insulin-secretion function. In an STZ-induced hyperglycemic mouse model, EGLP-1 increased β -cell mass in islets and improved β -cell function, possibly by reducing serum NEFA levels. In addition, EGLP-1 reduced serum NEFA levels by reducing the levels of pHSL. Therefore, EGLP-1 can be used as an adjuvant therapy to insulin to improve glycemic control and lipid metabolism in patients with T1DM.

Abbreviations

GLP-1 (glucagon-like peptide-1); EGLP-1 (a novel GLP-1 analogue); GLP-1R (glucagon-like peptide-1 receptor); STZ (streptozotocin;); DPP-IV (dipeptidyl peptidase IV); CG-HEK293 (co-transfected with HuGLP-1R and CRE plasmid in human embryonic kidney 293); IPGTT (intraperitoneal glucose tolerance test); TAG (Triglycerides); HSL (hormone sensitive lipase gene).

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L.J. designed and conducted the experiments. H.G. performed all the experiments and prepared the figures. Z.S., Q.Z., Y.W., S.T., Y.S., Y.X., Y.P. & Y.Z. helped with the animal experiments and collected the sample data. M.A. reorganized and reviewed the manuscripts. All authors contributed to the writing of the manuscript and discussed the research in the experimental period.

Disclosure Statement

The authors declare to have no competing financial interests.

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