Peptides 88 (2017) 46–54

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Research Paper

A novel GLP-1 analog, a dimer of GLP-1 via covalent linkage by a lysine, prolongs the action of GLP-1 in the treatment of type 2 diabetes

Yingying Pan[a,1], Siwei Shi[b,1], Xun Lao[a], Jinlong Zhang[a], Shiming Tan[a], Zirong Wu[a], Jing Huang[a,*]

[a] School of Life Science, East China Normal University, Shanghai, China
[b] School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China

ARTICLE INFO

Article history:
Received 16 August 2016
Received in revised form 28 October 2016
Accepted 17 November 2016
Available online 10 December 2016

Keywords:
GLP-1 analog
Dimerization
Type 2 diabetes
Animal experiment
Long-term treatment

ABSTRACT

GLP-1 is an incretin hormone that can effectively lower blood glucose, however, the short time of biological activity and the side effect limit its therapeutic application. Many methods have been tried to optimize GLP-1 to extend its in vivo half-time, reduce its side effect and enhance its activity. Here we have chosen the idea to dimerize GLP-1 with a C-terminal lysine to form a new GLP-1 analog, DLG3312. We have explored the structure and the biological property of DLG3312, and the results indicated that DLG3312 not only remained the ability to activate the GLP-1R, but also strongly stimulated Min6 cell to secrete insulin. The in vivo bioactivities have been tested on two kinds of animal models, the STZ induced T2DM mice and the db/db mice, respectively. DLG3312 showed potent anti-diabetic ability in glucose tolerance assay and single-dose administration of DLG3312 could lower blood glucose for at least 10 hours. Long-term treatment with DLG3312 can reduce fasted blood glucose, decrease water consumption and food intake and significantly reduce the HbA1c level by 1.80% and 2.37% on STZ induced T2DM mice and the db/db mice, respectively. We also compared DLG3312 with liraglutide to investigate its integrated control of the type 2 diabetes. The results indicated that DLG3312 almost has the same effect as liraglutide but with a much simpler preparation process. In conclusion, we, by using C-terminal lysine as a linker, have synthesized a novel GLP-1 analog, DLG3312. With simplified preparation and improved physiological characterizations, DLG3312 could be considered as a promising candidate for the type 2 diabetes therapy.

1. Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted from L cells of intestines following oral glucose administration [1,2]. GLP-1 effectively stimulates insulin release via exerting effects on β-cell through GLP-1 receptor (GLP-1R) to lower blood glucose [3,4]. Many evidences demonstrate that GLP-1 makes benefits in promoting β-cell proliferation [5,6] and inhibiting apoptosis [7,8]. Continuous administration of native GLP-1 for 6 weeks significantly decreases HbA1c in patients with type 2 diabetes [9]. Because of its glucose-dependent manner, GLP-1 makes the risk of hypoglycemia minimal [10]. Further researches have shown that GLP-1 can also effect on decreasing gastric emptying and reducing appetite [11–14]. However, GLP-1 is rapidly inactivated by dipeptidyl peptidase IV (DPPIV) in vivo and the resulting short biological half-life of 2–3 min limits its therapeutic use [15,16]. Based on the characters above, GLP-1, if optimized, can be developed into a promising pharmacological candidate for the treatment of type 2 diabetes. Thus many efforts have been made on developing long-acting GLP-1 analogs. The crucial point of designing new GLP-1 analogs is to prolong the stability in vivo [17]. Since the 8th amino acid of GLP-1 is the active site of DPP-IV, replacement or modification of the 8th amino acid was adopted by many GLP-1 analogs, such as BPI3006, which replaced unit -NHCO- with -CH(CF3)NH- of the 8th glutamic acid [18,19]. BPI3006 showed a potent stability to resist the cleavage of DPP-IV in vivo with 27.2% degradation after incubation with human plasma for 12 h. Another strategy is based on the incorporation of macromolecules, such as polyethylene glycol chains and circulating plasma proteins. For example, besides a substitution of Arg^{34} for Lys^{34}, liraglutide contains a C-16 free-fatty acid derivative, which is believed to promote the non-covalent binding of albumin and to protect the loss of liraglutide from renal filtration [20]. However, introducing

* Corresponding author.
E-mail address: jhuang@bio.ecnu.edu.cn (J. Huang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.peptides.2016.11.009
0196-9781/

macromolecular modifications usually involved lots of complicated synthesis and purification processes, which is money-consuming and unconducive to the quality control. Other strategies, like albumin fusion (albiglutide) [21] and Fc fusion (dulaglutide) [22], lead to the development of a new biological entity. In this case, higher clinical development risks and costs come along with the risk of the decrease of the biological activity, due to detrimental conjugation and/or steric hindrance issues [23]. For example, the fusion of albumin may result a reduced ability to activate the GLP-1 receptor [24].

It was reported that an intra-disulfide bond could improve the stability of GLP-1 [25]. In Ying Li's work, a cysteine and fifteen glutamic acids were added at C-terminal of GLP-1(7–37) to form a GLP-1 analog, GLP715a. GLP715a exhibited a remarkable improvement in stability in vitro compared to wild-type GLP-1 and showed a potent ability to stimulate β-cell secreting insulin. It indicated that the dimerization of GLP-1 monomers via disulfide bond could be a strategy for developing long-acting GLP-1 analogs. However, disulfide bonds between cysteine residues are often considered to be "weak link " in the protein or peptides, because it's easily broken down by reducing agents or disulfide reductase, which may limit the long-acting effect of GLP715a [26]. The typical bond dissociation energy of disulfide bonds is 268 KJ/mol, being about 25% stronger than disulfide bonds, amido bonds, the typical bonds in peptides, are thus much more stable [27,28].

Accordingly, an amido bond between two GLP-1 monomers could be an ideal strategy to stabilize GLP-1 in vivo. Thus in this study, a new structure of GLP-1 analog, DLG3312, employing a lysine to combine the C terminals of two monomers with its α-NH2 and ε-NH2 was designed and expected to resist degradation of DPP-IV in vivo. In our work the bioactivity of DLG3312 was investigated both in vitro and in vivo, liraglutide, marketed drug name as Victoza® was used as a positive control.

2. Materials and methods

2.1. Materials

The Streptozocin (STZ, purity ≥98%)was purchased from Sigma-Aldrich. Blood Glucose meter and filters were purchased form Roche. Liraglutide was purchased from Novo Nordisk (China) Pharmaceuticals Co., Ltd. Insulin ELISA kit was purchased from R&D SYSTEM China Co., Ltd. GLP-1R plasmid P_{CMV}-GLP-R-pA_{SV40}, cAMP response plasmid P_{CRE}-EYFP-pA_{SV40} and P_{CRE}-SEAP-pA_{SV40} were kindly donated by Professor Haifeng Ye. If not be specified, all other chemicals were purchased from Sigma-Aldrich.

2.2. Peptides

DLG3312 was synthesized and purified to >95% by Abbio Biochem Ltd., Shanghai. The amino acid sequence of DLG3312 and GLP-1(7–37) were shown in Fig. 1. DLG3312 and GLP-1(7–37) was diluted in phosphate buffer saline (PBS) at 20 μg/mL final concentration for detection of the secondary structure of peptides through circular dichroism method.

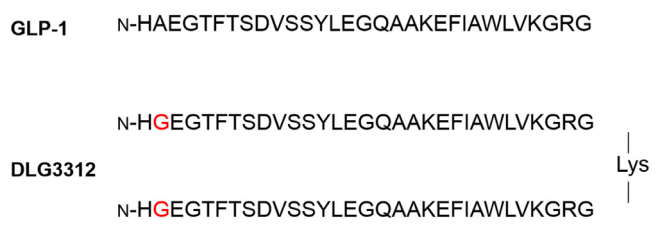

Fig. 1. The primary structure of GLP-1 and DLG3312. In DLG3312, a lysine was employed to combine the C terminals of two monomers covalently. The mutated G in DLG3312 was represented in red font. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Cells

Min6 cell line was purchased from Cell bank of Chinese academy of sciences and cultured with DMEM medium (4.5 g/L glucose) with 10% Fetal Bovine Serum (FBS) at 37 °C, 5%CO_2. HEK-293T cell line was purchased from Cell bank of Chinese academy of sciences and cultured with DMEM medium(4.5 g/L glucose) with 10% FBS at 37 °C, 5% CO_2.

2.4. Animals

The db/db mice (6–7weeks) were obtained from Animal Experiment Center of East China Normal University. The db/db mice were used for short-term experiments including glucose tolerance experiment, one-dose experiment, and long-term experiments.

Female C57BL/6 mice weighting 15 g, obtained from Animal Experiment Center of East China Normal University, were carried out as STZ-induced type 2 diabetes mellitus (T2DM) model mice. Firstly, female C57BL/6 wild-type mice were housed five per cage under 12-h light/dark cycle and fed with high-glucose food after injection of 50 mg/kg STZ for 5 consecutive days (once a day). After T2DM model mice had been established successfully, they were employed for long-term experiment. All animals were treated according to the standards for laboratory animals published by the People's Republic of China (GB14925-2001).

2.5. Serum stability in vitro

The stability of DLG3312 in the mouse serum was assayed according to our previous method [16]. In brief, DLG3312 was incubated with mice serum (0.8 mg/mL) at 37 °C for 12 h. The incubation was terminated by the addition of 5 μl of 20% trifluoroacetic acid (TFA). The samples were analyzed by high-performance liquid chromatography (HPLC) (Agilent, USA) with a C18 (4.6 × 250 mm) reverse-phase column (Kromasil, Sweden) eluted at a flow rate of 1.0 ml/min with a linear gradient of 0.1% TFA in water and 5%–95% acetonitrile for 20 min. Peptides were detected based on their absorbance at 220 nm and quantified by integration of their peak areas and comparison with internal standards.

2.6. GLP-1 receptor activation in vitro

HEK-293T cells were seeded in 24-well plate at 70% confluence and cultured to 80–90% confluence, followed by transiently transfected with GLP-1 receptor (GLP-1R) plasmid P_{CMV}-GLP-1R-pA_{SV40} and cAMP response plasmid P_{CRE}-EYFP-pA_{SV40}, expressing enhanced yellow fluorescent protein (EYFP) after activated, or P_{CRE}-SEAP-pA_{SV40}, expressing secreted alkaline phosphatase (SEAP) as the reporter gene, 6 h later, the medium was replaced by DMEM medium containing 10%FBS and different concentration of DLG3312 or GLP-1(7–37). After treating for 24 h, cells transfected with GLP-1R plasmid P_{CMV}-GLP-1R-pA_{SV40} and P_{CRE}-EYFP-pA_{SV40} were monitored with fluorescent inverted microscope for EYFP expression, which could reflect the activation of GLP-1R; cells transfected with GLP-1R plasmid P_{CMV}-GLP-1R-pA_{SV40} and P_{CRE}-EYFP-pA_{SV40} were removed to tubes and then centrifuged at 12, 000 rpm. The supernate was added with substrate of alkaline phosphatase and the mixture was read by microplate reader to measure the expression of SEAP at 405 nm, which could quantificationally reflect the activation of GLP-1R.