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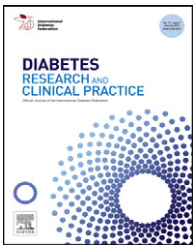

Peptide complex containing GLP-1 exhibited long-acting properties in the treatment of type 2 diabetes

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ABSTRACT

The multiple physiological characterizations of glucagon-like peptide-1 (GLP-1) make it a promising drug candidate for the treatment of type 2 diabetes. However, *in vivo*, the half-life of GLP-1 is short, which is caused by the degradation of dipeptidyl peptidase-IV (DPP-IV) and renal clearance. Thus, the stabilization of GLP-1 is critical for its utility in drug development. Peptides known as GLP-1 protectors are predicted to increase the half-life of GLP-1 *in vivo*. Protecting peptides are able to form stable complexes by non-covalent interactions with human GLP-1. In this study, the stability of the complex was investigated, and the physiological functions of the GLP-1/peptide 1 complex were compared to those of exenatide and liraglutide in animals. The results indicated that the GLP-1/peptide 1 complex remarkably raised the half-life of GLP-1 *in vivo* and showed better glucose tolerance and higher HbA_{1c} reduction than exenatide and liraglutide in rodents. Based upon these results, it is suggested that the GLP-1/peptide 1 complex might be utilized as a possible potent anti-diabetic drug in the treatment of type 2 diabetes mellitus.

1. Introduction

Glucagon-like peptide 1 (GLP-1) is a gut hormone released from intestinal L cells following oral glucose administration [1]. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) contribute to approximately 60–70% of the postprandial insulin response in healthy individuals [2]. However, in the absence of GIP, GLP-1 preserves the blood glucose lowering effect [3]. Therefore, GLP-1 is vital for insulin secretion, which suggests that it could have a potential application in therapeutic strategies for type 2 diabetes treatments [4]. GLP-1 serves as an incretin factor stimulating the secretion of insulin and reduces blood glucose both in normal subjects and in subjects with type 2 diabetes mellitus [5]. GLP-1 is a potent anti-hyperglycemic hormone, stimulating the secretion of insulin in a glucose-dependent manner and suppressing glucagon secretion, which minimizes the risk of hypoglycemia [6,7]. Interestingly, when the plasma glucose concentration is in the normal fasting range, GLP-1 does not stimulate insulin, which causes hypoglycemia [4]. It was found that GLP-1

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Abbreviations: AUC, area under the curve; DPP-IV, dipeptidylpeptidase IV; ELISA, enzyme-linked immunosorbent assay; GLP-1, glucagon-like peptide 1; IPGTT, intraperitoneally glucose tolerance test.

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restores the glucose sensitivity of pancreatic β-cells, which possibly depend on the increasing level of glucose transporter 2 (GLUT2) and glucokinase expression [8]. It was also confirmed that GLP-1 inhibits apoptosis (programmed cell death) in cells, consequently improving their survival [9].

The deficiency of GLP-1 secretion in type 2 diabetes suggested that GLP-1 acts as a promising potential therapy [10]. Evidence has demonstrated that the infusion of GLP-1 decreases blood glucose [11], and GLP-1 has been identified as playing a crucial role in the regulation of glucose metabolism. A recent report showed that GLP-1 has an exceptionally short half-life of less than 2 min *in vivo*, which is due to rapid degradation by the enzyme dipeptidyl peptidase IV (DPP-IV) [12]. This makes the therapeutic administration of GLP-1 seem impractical; thus, many efforts have focused on amending the pharmacokinetic properties of GLP-1 in a series of derivatives and analogues. Two GLP-1 analogues, exenatide and liraglutide, were approved by the FDA for the treatment of type 2 diabetes in 2005 and 2010, respectively [13]. Exendin-4 (exenatide is a synthetic exendin-4) shares 53% amino acid sequence similarity with GLP-1, which is a 39-amino acid peptide produced in the salivary glands of the Gila monster (*Heloderma suspectum*) [14]. No specific exendin-4 receptor was detected; the effect of exendin-4 is exerted through the GLP-1 receptor [15]. Liraglutide contains two modifications: a substitution of Arg^{34} for Lys^{34} and an attachment of a C-16 free-fatty acid derivative via a glutamoyl spacer to Lys^{26} [16]. The free-fatty acid derivative is supposed to promote the non-covalent binding of liraglutide and albumin [17]; accordingly, the absorption of liraglutide is delayed from the injection site, and clearance is also decreased [18]. In a LEAD6 study, Buse et al. compared the efficacy and safety of two GLP-1 analogues (exenatide and liraglutide) in the treatment of type 2 diabetes [19]. Liraglutide showed better glucose tolerance than exenatide and presented fewer side effects, such as nausea, than exenatide [19].

To avoid any possible immunotoxicity by non-mammalian products, it is preferable to employ human full-length GLP-1 as a tool for the treatment of type 2 diabetes in this study. Accordingly, the aim of this study is to stabilize GLP-1 *in vivo* by prolonging its half-life; an amphipathic peptide is the ideal candidate to achieve this goal. Amphipathic peptides contain the following two domains: a hydrophilic domain that interacts with negatively charged molecules and a hydrophobic domain that interacts with the cell membrane [20]. Accordingly, amphipathic peptides are employed as cell-penetrating peptides, but the uptake mechanism is still unclear and a limitation of the clinical utility of the cell-penetrating peptides. Divita et al. reported a secondary cell-penetrating peptide, CADY, which allowed the formation of a stable complex with siRNA via non-covalent interactions [21]. Nontoxic CADY-based technology has had a significant effect on the development of fundamental and therapeutic siRNA-based applications. In this research, CADY peptide acting as a protecting peptide was modified to achieve a stable complex of GLP-1 and protecting peptide. The physiological properties of the GLP-1/protecting peptide complex were investigated, including its stabilization, glucoregulatory and long-lasting anti-diabetic effects, in rodent and human serum.

2. Materials and methods

2.1. Materials

DPP IV enzyme (0.1 mg/ml; purity ~85%) was purchased from Sigma. Human GLP-1 (9-37) and goat anti-rat insulin ELISA kits were purchased from Phoenix Technology, Inc. The GLP-1 (7-37) ELISA kit was purchased from Millipore. A one-touch blood glucose meter and filters were purchased from Abbott. Other chemicals unless otherwise specified were purchased from Sigma.

2.2. Animals

All studies were performed with permits from the Animal Experiments Inspectorate, China. Male ZDF (fa/fa) rats, lean male ZDF rats and male Sprague Dawley (SD) rats were obtained from Shanghai Laboratory Animal Co. (SLAC), China Academy of Sciences (Shanghai, China).

2.3. Peptide synthesis

GLP-1 (HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR) and two protecting peptides (peptide 1: GLWWKAWWKAWWKSLWWRKRKRKA; peptide 2: GLWWKVWWKVWWKSLWWRKRLRKA) were purchased from Sangon Biotech Co. (Shanghai; HPLC-purified; purity >90%, identified by MS). The freeze-dried peptides were weighed and dissolved in pure water to make 10 mg/ml stock solutions for further analysis. Exenatide and liraglutide were kind gifts from Dr. Wei Liu in Tianjin General Hospital.

2.4. Preparation of blood samples

Human whole blood from healthy individuals and rat blood samples were directly drawn into P800*, K_2EDTA tubes (BD, Franklin Lakes, NJ) by venipuncture. Blood sample was immediate centrifugation for 20 min at 11,903 × *g* for obtaining serum. The serum samples were stored at −80 °C for further use.

2.5. Analysis of the mixture of GLP-1 and protecting peptides by HPLC

GLP-1 and protecting peptides (peptide 1 or peptide 2) were mixed at various ratios and then incubated at 25 °C for 5 min. Different amounts of peptides were applied: 100 μg/ml, 500 μg/ml, 1 mg/ml and 2 mg/ml, while the concentration of GLP-1 remained at 100 μg/ml. The final ratios of GLP-1 to peptide were 1:1; 1:5; 1:10 and 1:20, respectively. Mixtures (10 μl) were analyzed by a Surveyor HPLC system through a C18 analytical column. The column was eluted at a flow rate of 0.5 ml/min in a gradient mode with the mixture of mobile phase A (H_2O + 40% acetonitrile + 0.1% trifluoroacetic acid) and mobile phase B (acetonitrile + 0.1% trifluoroacetic acid). Mobile phase A was eluted for 10 min, and thereafter, mobile phase B was increased from 40% to 100% in a 60-min period. HPLC analyses were performed at ambient temperature and the UV detection wavelength was set at 220 nm. Ten microliter