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Rapid communication

Glucagon-like peptide-1(1–37) can enhance blood glucose homeostasis in mice

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is produced by the posttranslational processing of proglucagon and acts as a regulator of various homeostatic events. No blood glucose regulation role of GLP-1(1–37) has previously been identified. However, our findings in this study clearly showed that GLP-1(1–37) could lower blood glucose levels both in normal and diabetic mice. In vitro stability analysis demonstrated that GLP-1(1–37) was more stable than GLP-1(7–37), with 94.7% of the initial amount of peptide left after a 4 h exposure to mouse serum. Moreover, GLP-1(1–37) was confirmed to be a highly potent agonist of the GLP-1 receptor (GLP-1R) by measuring the expression of the luciferase reporter gene expression in transiently transfected human embryonic kidney (HEK293) cells. Unlike the glucose lowering effect of GLP-1(7–37), the glucose-lowering effect of GLP-1(1–37) could not be blocked by the GLP-1R antagonist exendin(9–39), suggesting that GLP-1(1–37) might activate the GLP-1R via a different mechanism. Therefore, our findings suggest that GLP-1(1–37) could be a potential therapeutic drug for the treatment of type 2 diabetes in the future.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is produced by the posttranslational processing of proglucagon and is secreted from intestinal L-cells in two bioactive forms, GLP-1(7–37) and GLP-1(7–36) amide, the predominant circulating active form. Both peptides have the same biological activity and half-life [1–3]. GLP-1 can stimulate glucose-dependent insulin secretion and inhibit glucagon secretion to induce a decrease in the postprandial blood glucose level [4,5]. GLP-1 can also induce β-cell proliferation and islet neogenesis and inhibit β-cell apoptosis [1,6]. In addition to these functions, GLP-1 can inhibit gastrointestinal motility and reduce appetite [7]. A prominent feature of GLP-1 is its glucose-dependent insulinotropic action; unlike the bioactivity of incretin hormone gastric inhibitory polypeptide, the bioactivity of GLP-1 is preserved in the diabetic state [8]. Thus, GLP-1 is a potent drug for treating type 2 diabetes [9,10].

The untruncated GLP-1(1–37) is a 37-amino-acid hormone that is secreted from the pancreas and to a lesser extent from the intestinal cells [11]. It can be cleaved by an unidentified protease to generate truncated GLP-1(7–37) in the intestinal L cells [12]. Most studies have focused on the effects of the truncated GLP-1 form, and the role of the untruncated GLP-1(1–37) form remains incompletely understood. Recently, it has been demonstrated that GLP-1(1–37) inhibits the chemokine-induced migration of human CD4-positive lymphocytes [13], and GLP-1(1–37) also has been shown to convert intestinal epithelial cells into insulin-producing cells [14]. However, the role of GLP-1(1–37) in reducing the blood glucose level is still unclear. Therefore, in the present study, we examined the regulation of the in vivo blood glucose levels by GLP-1(1–37).

2. Materials and methods

2.1. Materials

All enzymes used for molecular cloning were purchased from Takara (Shanghai, China). The GLP-1R plasmid and the CRE-luciferase plasmid (CRE: cAMP-responsive element) were gifts from the National Center for Drug Screening (Shanghai, China). *Escherichia coli* BL21(DE3) cell line was purchased from Merck (Germany). The peptides GLP-1(7–37) (purity, 98%) and exendin(9–39) (purity, 98%) were synthesized by ChinaPeptides Corporation (Shanghai, China). GLP-1(1–37) used for the GLP-1R activation assay was synthesized by Chinese Peptide Corporation (Hangzhou, China). Normal Kunming (KM) mice (female, 10 weeks old, 20–25 g) and db/db diabetic mice (female, 16 weeks old, 40–50 g) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China) and housed with a 12-h:12-h light–dark cycle with ad libitum access to food and water. All procedures involving animals were approved by the Science and Technology Commission of Shanghai Municipality.

2.2. Cloning, expression and purification of GLP-1(1–37)

The gene encoding GLP-1(1–37) was amplified from pET32a-GLP-1(7–37) by PCR using a forward primer containing the sequence of

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GLP-1(1–6) (5′-CATGATGAATTTGAACGCCATGCCGAAGGCACCTTTA CC-3′) and a reverse primer (5′-GGAAGCTTGTTAGCCTCTGCCTTT CACCAGCC-3′). The final PCR product was identified by sequencing, digested with *Bgl* II and *Hind* III and then cloned into the corresponding sites of the pET32a(+) vector to obtain a recombinant plasmid pET32a-GLP-1(1–37). The protein expressed from this plasmid contains an N-terminal His6-tag for purification and an enterokinase site for cleavage. *E. coli* BL21(DE3) cells transformed with recombinant plasmid pET32a-GLP-1(1–37) were cultured in Luria-Bertani medium at 37 °C. After isopropylthio-β-D-galactoside (IPTG) induction for 4 h, the fusion proteins were purified by Ni^{2+}-affinity chromatography and cleaved by enterokinase. The digested product was further purified using Ni^{2+}-affinity chromatography [15]. The proteins were analyzed by SDS-PAGE, and the concentration was determined by the BCA kit according to the manufacturer's instructions (Biocolor Inc., Shanghai, China).

2.3. Cell culture

The HEK293 cell line was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37 °C in 5% CO_2.

2.4. Serum stability

GLP-1(1–37) and GLP-1(7–37) were incubated with mouse serum (final sample volume, 200 μl; final peptide concentration, 1 mg/ml) for 0 h and 4 h at 37 °C, respectively. The incubation was terminated by the addition of 5 μl of 20% trifluoroacetic acid (TFA) [16]. The samples were analyzed by high-performance liquid chromatography (HPLC) (Agilent, USA) with a C_{18} (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.

2.5. Measurement of luciferase activity

HEK293 cells (5×10^4) were seeded in a 96-well plate and transiently cotransfected with the GLP-1R plasmid and the CRE-luciferase reporter plasmid using Lipofectamine 2000 (Invitrogen, USA). After a 48 h transfection, different concentrations of GLP-1(1–37) or GLP-1(7–37) were added, and the cells were incubated for 5 h. The cells were harvested for a luciferase assay using a luciferase assay kit according to the manufacturer's instructions (Promega, USA). A dose–response equation $\%\ Response = \frac{L_{Sample}-L_{Blank}}{L_{ex-4}-L_{Blank}} \times 100\%$ was used to determine the bioactivity of GLP-1 analogs. L_{Sample} means for the detection signal of samples; L_{Blank} means for the detection signal of empty wells; L_{ex-4} means for the detection signal of 1000 nM positive control GLP-1.

2.6. Glucose-lowering effect in vivo

The bioactivities of GLP-1(1–37) were determined using an intraperitoneal glucose tolerance test (IPGTT) in KM mice. The mice were fasted for 16 h and then treated with GLP-1(1–37) at doses of 6, 12 and 24 nmol/kg in combination with glucose at a dose of 4 g/kg in KM mice via intraperitoneal injection. Blood was collected from the tail vein at 0, 15, 30, 45, 60, 90 and 120 min after glucose and protein administration. The blood glucose levels were measured by the glucose oxidase method using a glucose meter (MicroSense Inc., Shanghai, China), and the areas under the curve (AUC) for the glucose levels were measured using GraphPad Prism. The IPGTT was also performed both in normal mice and diabetic mice to determine the duration of the efficacies of GLP-1(1–37) (24 nmol/kg) and GLP-1(7–37) (24 nmol/kg). The glucose was administrated at a dose of 4 g/kg in normal mice or 1 g/kg in db/db mice at 0 and 60 min after GLP-1(1–37) or GLP-1(7–37) administration.

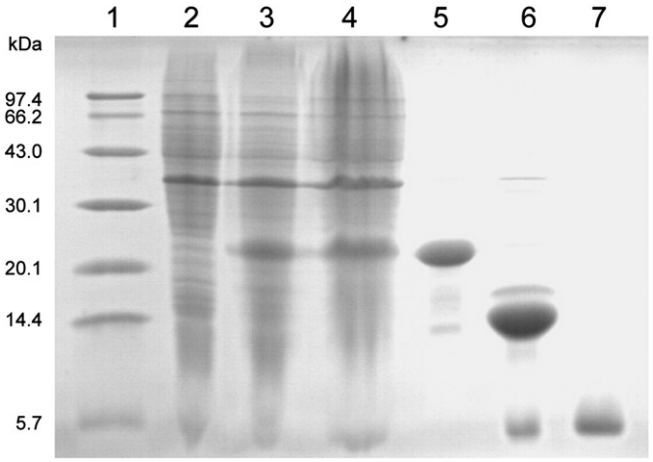

Fig. 1. The purification of recombinant GLP-1(1–37) protein. *Escherichia coli* BL21(DE3) cells containing pET32a-GLP-1(1–37) were crushed with an ultrasonic cell crusher. Lane 1, protein molecular weight markers; lane 2, total cell lysate before IPTG induction; lane 3, total cell lysate after IPTG induction; lane 4, supernatant of the total cell lysate; lane 5, purified fusion protein eluted from the Ni^{2+}-affinity chromatography column; lane 6, purified protein treated with enterokinase; lane 7, purified recombinant GLP-1(1–37) after the removal of thioredoxin and the His6-tag.

2.7. Effect of GLP-1(1–37) with exendin(9–39) on blood glucose-lowering

The normal KM mice were fasted for 16 h before the administration (i.p.) of GLP-1 and glucose. GLP-1(1–37) (25 nmol/kg) with or without exendin(9–39) (250 nmol/kg) was given in combination with glucose (4 g/kg). GLP-1(7–37) (25 nmol/kg) with or without exendin(9–39) (250 nmol/kg) was also administrated in combination with glucose (4 g/kg). The control group was treated with saline (NaCl, 9 g/l) and glucose (4 g/kg). The IPGTT was carried out at 0, 15, 30 and 60 min after glucose and protein administration, and the blood glucose levels were measured as described above.

2.8. Statistical analysis

The data were presented as the means ± S.E.M. The statistical significance of the data was evaluated using Student's *t*-test. For all statistical tests, P values <0.05 were considered as statistically significant.

3. Results

3.1. Expression and purification of GLP-1(1–37)

The GLP-1(1–37) gene was cloned into the pET32a(+) expression vector, which contains sequences for an N-terminal thioredoxin, a hexahistidine (His6) tag for purification and an enterokinase site for cleavage. The recombinant plasmid pET32a-GLP-1(1–37) was transformed into the *E. coli* strain BL21(DE3). The expressed fusion protein carrying thioredoxin and the His6-tag was purified via Ni^{2+}-affinity chromatography. After enterokinase cleavage, the protein fragment containing thioredoxin and the His6-tag was removed by Ni^{2+}-affinity chromatography. The final pure GLP-1(1–37) protein was obtained

Table 1
Stability of peptide in mouse serum in vitro.

Time of incubation	The initial amount of peptide left	
	GLP-1(1–37)	GLP-1(7–37)
0 h	98.4%	97.0%
4 h	94.7%	2.3%

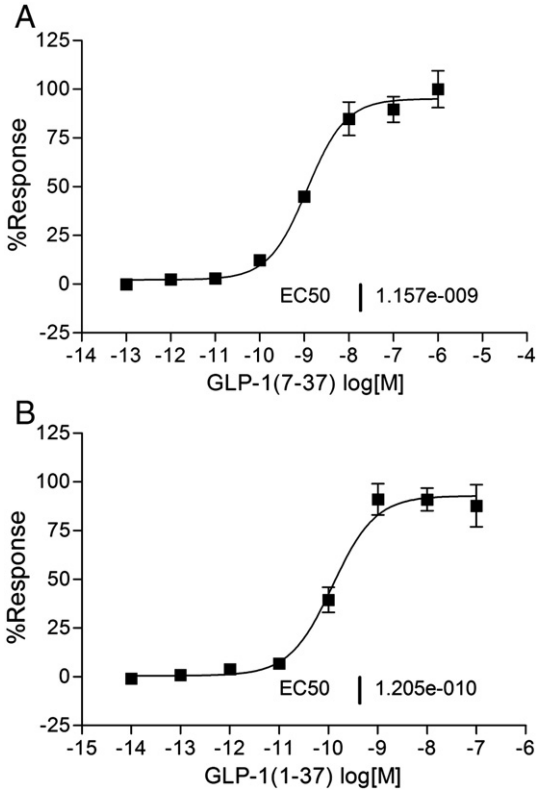

Fig. 2. Luciferase expression in HEK293 cells co-transfected with GLP-1R and CRE-luciferase plasmids. The GLP-1 receptor activation activities of GLP-1(7–37) (A) and GLP-1(1–37) (B). HEK293 cells were incubated with the indicated concentrations of GLP-1(1–37) or GLP-1(7–37) for 5 h before measuring the luciferase activity.

in the flow-through of the final Ni^{2+}-affinity chromatography step (Fig. 1).

3.2. The stability of GLP-1(1–37) in mouse serum

As shown in Table 1, GLP-1(1–37) was more stable than GLP-1(7–37) in mouse serum, with 94.7% of the initial amount of peptide remaining after a 4 h incubation. However, only 2.3% of the GLP-1(7–37) remained.

3.3. GLP-1R activation in vitro

A transient transfection system, involving GLP-1R-expressing HEK293 cells, is used to determine the bioactivity of GLP-1 analogs. In this system, the activation of the GLP-1R can be monitored by measuring the expression level of the luciferase reporter gene, because GLP-1R analogs induce the up-regulation of cAMP, which in turn, triggers the cAMP-dependent expression of the luciferase reporter gene [17]. Therefore, we used this system to confirm that GLP-1(1–37) could induce GLP-1R-mediated signaling in HEK293 cells coexpressing the GLP-1R and the CRE-luciferase receptor. As shown in Fig. 2, both GLP-1(1–37) and GLP-1(7–37) strongly stimulated luciferase reporter gene expression in a dose-dependent manner in HEK293 cells transfected with the GLP-1R plasmid and the CRE-luciferase reporter plasmid. Surprisingly, GLP-1(1–37) displayed an EC_{50} value of 0.12 nmol/l (nM), which was approximately 10-fold less than that of GLP-1(7–37) (EC_{50} value of 1.16 nM). This result suggests that GLP-1(1–37) is a highly potent agonist of GLP-1R.

3.4. Blood glucose-lowering effect

To determine the therapeutic efficacy of GLP-1(1–37) in vivo, an IPGTT was performed in normal KM mice. The results showed that GLP-1(1–37) decreased glycemic excursion in a dose-dependent

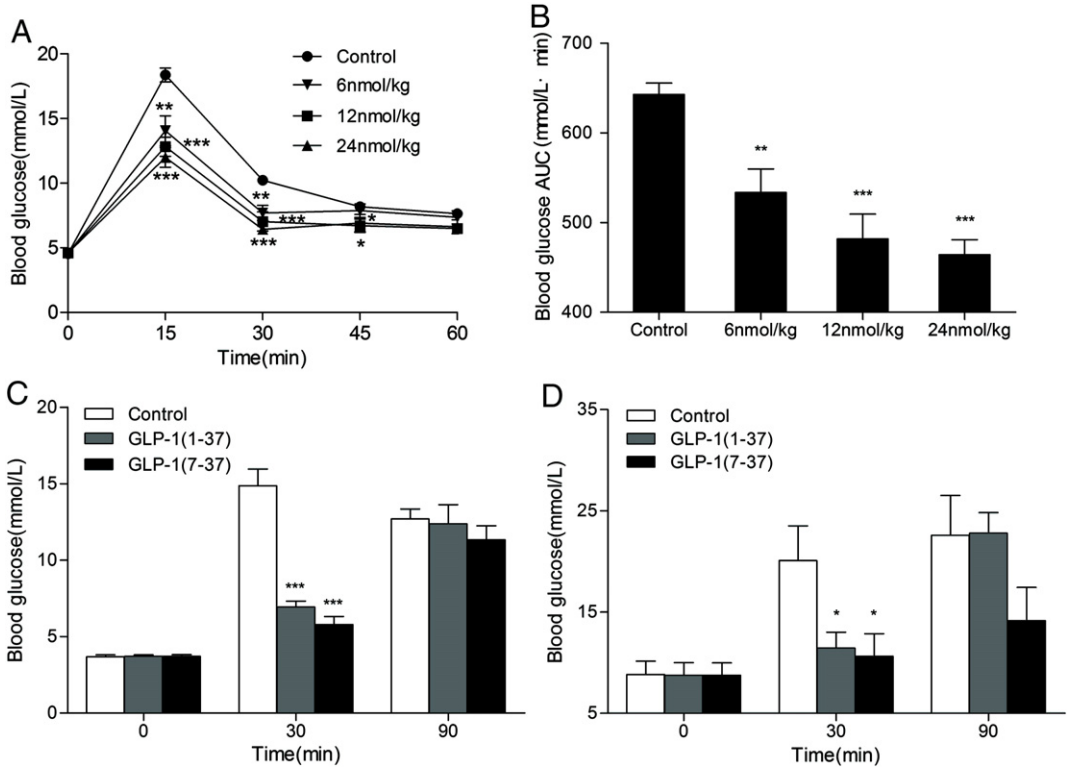

Fig. 3. GLP-1(1–37) reduced the blood glucose level in vivo. The IPGTT was performed with the administration of GLP-1(1–37) at doses of 6, 12 and 24 nmol/kg, respectively. The blood glucose levels were measured for 60 min (A), and the AUC for blood glucose was calculated (B). The efficacy durations of GLP-1(1–37) (24 nmol/kg) and GLP-1(7–37) (24 nmol/kg) were determined in KM mice (C) and db/db mice (D). Values are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the control.

manner (Fig. 3A). The areas under the blood glucose curve (AUCs) in the treated groups were also significantly lower than that of the control group (Fig. 3B). This result indicates that GLP-1(1–37) could lower the blood glucose level in vivo.

The IPGTT was performed both in normal and diabetic mice to determine the duration of the efficacies of GLP-1(1–37) and GLP-1(7–37) in the treatment of hyperglycemia. As shown in Fig. 3C and D, after the injection of GLP-1(1–37) (24 nmol/kg) or GLP-1(7–37) (24 nmol/kg), the two proteins showed blood glucose-lowering effect at 30 min, but they did not decrease the glucose levels at 90 min.

3.5. Blood glucose-lowering effect in the presence of exendin(9–39)

The administration of GLP-1(1–37) or GLP-1(7–37) markedly decreased blood glucose levels at 15 min and 30 min compared with the control group (Fig. 4). The glucose-lowering effect of GLP-1(7–37) could be blocked by the simultaneous injection of the GLP-1R antagonist exendin(9–39), whereas the glucose-lowering effect of GLP-1(1–37) remained when co-injected with exendin(9–39) (Fig. 4A). The AUC data showed the same result (Fig. 4B).

4. Discussion

GLP-1 is synthesized by posttranslational processing of proglucagon in the intestine and pancreas. Tissue-specific processing leads to the formation of several similar analogs. The truncated GLP-1(7–37) as a mature peptide has been shown to lower blood glucose levels and could be used as a therapeutic drug to treat type 2 diabetes. In contrast to GLP-1(7–37), the function of GLP-1(1–37) is still largely unknown. Previous studies found that GLP-1(1–37) could induce insulin production in development and to a lesser extent, adult intestinal epithelial cells in vitro and in vivo and implantation of GLP-1(1–37)-induced insulin-producing intestinal epithelial cells could restore glucose homeostasis in diabetic mice [14]. In addition, GLP-1(1–37) showed that it could inhibit chemokine-induced migration of human CD4-positive lymphocytes, which potentially contribute to the modulation of vascular inflammation in diabetic patients [13]. All these studies suggest that GLP-1(1–37) is a functional peptide rather than a nonfunctional propeptide. Howerer, the role of GLP-1(1–37) in lowering blood glucose levels was less clear. Our results in this study clearly show that GLP-1(1–37) enhances blood glucose homeostasis in both normal and diabetic mice, thus reducing blood glucose levels in vivo.

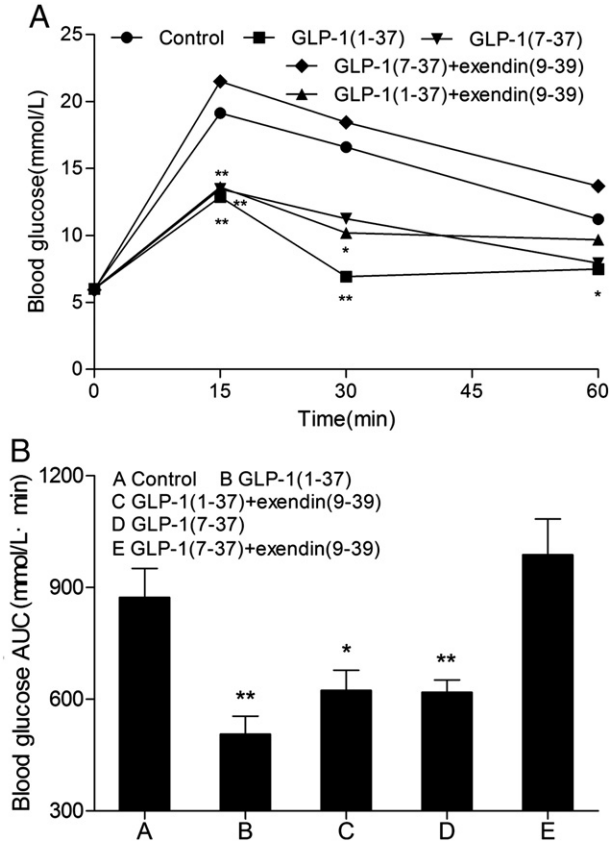

Fig. 4. The effect of treatment with exendin(9–39) on the glucose-lowering action of GLP-1(1–37) in normal KM mice. The concentrations of blood glucose were measured before (t = 0) and up to 60 min after the i.p. administration of saline (NaCl, 9 g/l), GLP-1(1–37) (25 nmol/kg), GLP-1(7–37) (25 nmol/kg), GLP-1(1–37) + exendin(9–39) or GLP-1(7–37) + exendin(9–39) (25 nmol/kg + 250 nmol/kg) combined with glucose (4 g/kg) at 0 min. The blood glucose levels were measured for 60 min (A), and the AUC for blood glucose was calculated (B). Values are the means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. the control.

It is reported that GLP-1(1–37) is a highly potent agonist of the GLP-1R by a transient transfection system involving GLP-1R-expressing HEK293 cells. Furthermore, the result of the antagonistic study in vivo showed that the activity of recombinant GLP-1(1–37) could not be blocked by GLP-1R antagonist exendin(9–39) and that GLP-1(1–37) still could reduce the blood glucose level, whereas the blood glucose-lowering effect of GLP-1(7–37) was completely abolished by exendin(9–39). Data on GLP-1(1–37) indicate that GLP-1(1–37) activates the GLP-1R and lowers the blood glucose level via a different mechanism compared to native GLP-1. To understand the mechanism by which GLP-1(1–37) lowers blood glucose, future studies are needed and Protein Kinase A (PKA) activity could be the main target to study.

It is well known that the first two amino acids at the N-terminus of truncated GLP-1(7–37) can be cleaved by the enzyme dipeptidyl peptidase IV (DPP-4), which results in a very short half-life of only 1–2 min for GLP-1(7–37) [18–20]. Therefore, in this study the stability of GLP-1(1–37) in mouse serum was evaluated by HPLC, and the results showed that GLP-1(1–37) was much more stable than GLP-1(7–37) in mouse serum. According to the literature [21], proconvertase 1 (PC1) could be the proconvertase which is responsible for the cleavage of the proglucagon processing residues in the intestine's L cells in vivo. However, when PC1 was incubated with GLP-1(1–37), no conversion to the intact GLP-1(7–37) was initially found [19]. Only when PC1 in large amounts was incubated with GLP-1(1–37) for 16 h, there was a partial conversion observed [22]. Although in this study we showed that GLP-1(1–37) incubated with mouse serum in vitro was stable, whether GLP-1(1–37) was conversed by proconvertase to GLP-1(7–37) which induced hypoglycemic activity in vivo, was still unknown. Moreover, the proconvertases are closely associated to the membrane of the secretory granules, which indicates that they are not likely to be released to the circulation [23]. Therefore, the mechanism of GLP-1(1–37) on regulating blood glucose levels is still not clear and more physiological studies, such as insulin secretion on isolated islets, cAMP production, in vitro antagonistic studies need to be further carried out in the future work.

In conclusion, our results clearly demonstrate that recombinant GLP-1(1–37) is a GLP-1R agonist and has a higher level of serum stability than native GLP-1. These properties confer GLP-1(1–37) the ability to lower blood glucose levels in mice, suggesting that this protein could be used as a potential drug for the treatment of type 2 diabetes.

Acknowledgments

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