A mutated glucagon-like peptide-1 with improved glucose-lowering activity in diabetic mice

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Keywords
diabetes; glucagon-like peptide-1; site-directed mutagenesis; streptozotocin

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Received May 22, 2012
Accepted October 11, 2012
doi: 10.1111/jphp.12011

Abstract
Objectives The aim of this study was to characterize the conformation and potency of a mutated glucagon-like peptide-1 (mGLP-1), and evaluate its glucose-lowering activity in diabetic mice.

Methods Spectroscopy techniques were employed to characterize the conformation of mGLP-1. Glucose tolerance test was performed to determine the potency of mGLP-1 in vivo. A mouse model in which diabetes was induced by multiple low doses of streptozotocin was established to evaluate the glucose-lowering activity of mGLP-1.

Key findings Compared with native GLP-1, mGLP-1 had a similar conformation and an enhanced potency in vivo. In diabetic mice, mGLP-1 displayed a significantly improved glucose-lowering activity as judged by fasting glucose and insulin, oral glucose tolerance test, beta cell function analysis and histochemical analysis.

Conclusions Collectively, mGLP-1 possesses an improved glucose-lowering activity in vivo and therefore can be recognized as a potential candidate for the future development of anti-diabetic drugs.

Introduction
The prevalence of diabetes is increasing globally along with lifestyle and diet-structure change.[1,2] Even worse, its complications, such as cardiovascular diseases, are becoming one of the major causes of mortality throughout the world.[3] Many anti-diabetic drugs are available, but when using these current drugs many diabetic patients cannot control their blood glucose precisely for a long term without developing vascular complications.[4] Therefore, development of new drugs to treat diabetes and to prevent its complications is necessary and urgent.

Glucagon-like peptide-1 (GLP-1) is a promising molecule for anti-diabetic drug development. As an endogenous glucose-lowering peptide, GLP-1 exerts its glucose-lowering effect through multiple pathways including increasing insulin secretion and slowing down gastric emptying.[5,6] Moreover, it can increase pancreatic beta cell mass via modulating differentiation, proliferation and apoptosis.[7,8] Additionally, its risk of inducing hypoglycaemia is low because its intracellular signal transduction is glucose dependent.[5] Due to these benefits, GLP-1 was regarded as an ideal candidate for anti-diabetic drug development.[9] Unfortunately, however, GLP-1 has a poor stability in vivo, which limits its half-life to 5 min in human.[9] Due to this limitation, native GLP-1 is not suitable for the treatment of diabetes.

Many technologies, including chemical modification, fusion protein construction and site-directed mutagenesis, have been attempted to improve the anti-diabetic effect of GLP-1.[10-13] Among these technologies, site-directed mutagenesis has been successfully used on insulin analogues and is more attractive.[14,15] Using site-directed mutagenesis we constructed a mutated GLP-1 (mGLP-1 (A2G, K28R)) in a previous study and optimized its recombinant production.[16] This mutated peptide showed a dose-dependent acute glucose-lowering effect in normal mice.[16] However, the glucose-lowering activity of mGLP-1 in a diabetic animal model remains to be evaluated. In this study we evaluated its glucose-lowering activity in a mouse model of
diabetes induced by multiple low doses of streptozotocin (STZ), and also investigated its conformation and potency in vivo.

Materials and Methods

Materials

Native GLP-1 was purchased from TASH biotechnology company (Shanghai, China). Chinese Kunming (KM) mice were purchased from Experimental Animal Center of Nanjing Military Region (Nanjing, China) and the use of animals was approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (No. of certification: SYXK (Su)-2007-0025; date of approval: 05 July 2007). STZ was purchased from Sigma-Aldrich (St Louis, MO, USA). Glucose determination kit (No. F006, hexokinase assay) and insulin determination kit (No. H203, enzyme-linked immunosorbent assay) were purchased from Jiancheng biotechnology company (Nanjing, China). All other chemical reagents were obtained from standard commercial sources and were of analytical grade.

Circular dichroism and fluorescence emission spectra

Peptide dissolved in sodium phosphate buffer (10 mM, pH 7.2) at 0.1 mg/ml was used for circular dichroism (CD) spectrum determination on Jasco J-715 spectropolarimeter (Jasco Analytical Instruments, Easton, MD, USA) with K-2D program. The wavelength range was set at 190–260 nm under constant nitrogen purging according to the manufacturer’s instructions. The same method was used for sample preparation for fluorescence emission (FE) spectrum determination in which the temperature and excitation wavelength were set at 25°C and 280 nm, respectively.

Potency test in vivo

KM male mice were fasted overnight before this test. These mice were divided into three groups (n = 6 for each group), including control group (control + saline), diabetic model group (diabetes + saline), GLP-1-treated group (diabetes + GLP-1) and mGLP-1-treated group (diabetes + mGLP-1). These mice were injected intraperitoneally with saline (control group) or STZ (50 mg/kg) daily for 5 days. Treatment was started 3 days after the last injection of saline or STZ. Peptide-treated groups were injected intraperitoneally with GLP-1 or mGLP-1 at a dose of 25 nmol/kg daily for 14 days. Control and diabetic groups were injected intraperitoneally with the same volume of saline. Food and water intake was determined daily by weighing the food and water supplied and remaining in each cage. Body weight was measured weekly. Fasting glucose was determined weekly by using the same method as described above.

Oral glucose tolerance test

Mice were fasted overnight before the oral glucose tolerance test (OGTT), which was performed 1 day after the last injection of peptides or saline. Glucose dissolved in saline was administered by oral gavage at a dose of 1 g/kg, and blood samples were collected at predetermined time points (0, 30, 60 and 120 min). Blood glucose was measured using the same method as described above.

Insulin level and HOMA-β

Mice were sacrificed by carbon dioxide after 14 days of treatment. Plasma samples were collected from the heart and stored at −80°C. Blood insulin was measured by a commercial insulin assay kit (enzyme-linked immunosorbent assay). Homeostasis model assessment beta cell function index (HOMA-β) was calculated based on fasting glucose and insulin using the following formula: HOMA-β = 20 × insulin level/(fasting glucose − 3.5).

Histochemical study

Neutral buffered formalin (10%) was utilized for tissue fixation. Organs were dehydrated and embedded into paraffin. Tissue sections were cut at 6 μm and stored at room temperature. Hematoxylin and eosin (H&E) staining was performed following a standard protocol. Antigen retrieval for insulin immunohistochemistry (IHC) staining was conducted by using citrated buffer (20 mM, pH 6.0). After
antigen retrieval, the sections were incubated in 3% hydrogen peroxide for 15 min to inactivate the endogenous peroxidase, and thereafter were blocked in 10% normal serum for 1 h at room temperature before incubated with the antibody of insulin at 4°C overnight. Finally these sections were stained by diaminobenzidine. All of these tissue sections were examined by an optical microscope.

**Statistics**

The data are expressed as means ± SD for each group. Statistical analysis was performed by using Kruskal–Wallis test (with Dunn’s post-hoc tests). A value of \( P < 0.05 \) was considered significant.

**Results**

**CD spectrum and FE spectrum**

CD and FE spectra were examined to investigate whether the conformation of mGLP-1 was changed after mutation. Both peptides had similar CD spectra (Figure 1a), with an \( \alpha \)-helix content of \( \sim \)28% as analysed by K-2D program, suggesting they had a similar secondary structure composition. FE spectrum determination was performed to further analyse the local conformation around W25 in mGLP-1. Our data showed the emission profiles were almost identical for both peptides, with the maximum emission wavelength at \( \sim \)353 nm (Figure 1b), indicating that both peptides had similar local conformation around their W25. Collectively, these data demonstrated the conformation of mGLP-1 was maintained after site-directed mutation.

**Potency test in vivo**

A tolerance test was performed to study the potency of mGLP-1 in vivo. Consistent with our previous study, both peptides displayed an acute glucose-lowering effect in normal mice, with a glucose reduction of \( \sim \)40% and \( \sim \)44% for GLP-1 and mGLP-1, respectively (Figure 2a). No significant difference was observed between their respective acute glucose-lowering activities (Figure 2a). However, at 120 min, mGLP-1 reduced blood glucose by \( \sim \)23% while native GLP-1 completely lost its activity (Figure 2b). These data clearly demonstrated that the potency of mGLP-1 was greatly enhanced in vivo by the site-directed mutation.

**Food intake, water intake, body weight and fasting glucose**

Compared with normal mice, STZ-induced diabetic mice consumed more food and water, with an elevation of \( \sim \)0.5 fold and \( \sim \)3.4 fold in food intake and water intake, respectively (Figure 3a, b). Both mGLP-1 treatment and GLP-1 treatment markedly reduced these elevations (Figure 3a, b). Compared with GLP-1, mGLP-1 was more effective in reducing food intake and water intake. Diabetic mice treated with peptide or saline had a lower body weight than control mice treated with saline (Figure 2c). Finally, the fasting glucose level of the diabetic mice was 20.8 ± 3.1 (mmol/l) for the saline-treated group, 15.8 ± 2.1 (mmol/l) for the GLP-1-treated group and 12.8 ± 1.9 (mmol/l) for the mGLP-1-treated group (Figure 3d). Glucose homeostasis of the diabetic mice was greatly improved by mGLP-1 treatment.

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**Figure 1** Profiles of circular dichroism (CD) and fluorescence emission (FE) spectra. (a) CD spectra of GLP-1 and mGLP-1. (b) FE spectra of GLP-1 and mGLP-1.
OGTT in diabetic mice

OGTT was performed to further investigate the effect of mGLP-1 treatment on glucose homeostasis in diabetic mice. Compared with control mice treated with saline, diabetic mice treated with saline had an impaired glucose homeostasis: the glucose peak level in OGTT was 12.5 ± 0.6 (mmol/l) for control mice and 28.4 ± 1.1 (mmol/l) for diabetic mice (Figure 4a). Compared with that of control mice, the area under the curve (AUC; for glucose concentration vs time) of the diabetic mice was elevated by ~1.4 fold (Figure 4b). The impaired glucose homeostasis was greatly improved by mGLP-1 treatment, with a reduction of ~37% and ~40% in glucose peak level and AUC, respectively (Figure 4a, b). GLP-1 treatment had a similar but less pronounced effect, with a reduction of ~16% in glucose peak level and ~21% in AUC (Figure 4a, b).

Insulin level and HOMA-β

Blood insulin was measured to further investigate the effects of mGLP-1 treatment in diabetic mice. Diabetic mice treated with saline had a lower blood insulin concentration than control mice, with a reduction of ~69% (Figure 4c). The reduced blood insulin was greatly elevated by peptide treatment, with an elevation of ~0.4 fold for GLP-1 treatment and ~0.9 fold for mGLP-1 treatment (Figure 4c). HOMA-β was calculated to investigate the impact of mGLP-1 treatment on pancreatic beta cell function. STZ significantly reduced HOMA-β by 92% while GLP-1 treatment and mGLP-1 treatment elevated this value by ~0.9 fold and ~2.6 fold, respectively (Figure 4d). However, diabetic mice treated with peptide had a lower HOMA-β than control mice treated by saline, suggesting that their beta cell function was only partially recovered after these peptide treatments.

Histochemical study

H&E staining in the tissue sections of the pancreas, liver and kidney from STZ mice was performed to further investigate the effects of mGLP-1 treatment. In the pancreas, STZ caused tissue damage and decreased islet size, and these damages were partially repaired by mGLP-1 treatment (Figure 5). In the liver and kidney, which are major organs for drug metabolism and elimination, neither STZ nor peptides caused obvious morphological changes (Figure 5). Insulin IHC staining was performed to detected insulin level in the pancreatic islets. Diabetic mice had a lower level of insulin in the islets compared with control mice, and mGLP-1 treatment partially recovered its level (Figure 5), consistent with the result of blood insulin determination.

Discussion

In this study we characterized the conformation and potency of mGLP-1, and evaluated its glucose-lowering activity in diabetic mice. Compared with native GLP-1, mGLP-1 had a similar conformation and its potency was greatly enhanced in vivo (Figures 1 and 2). In diabetic mice, mGLP-1 had a significantly improved glucose-lowering activity as judged by fasting glucose and insulin, OGTT, HOMA-β and the results of histochemistry (Figures 3–5).

The structure–activity relationship of GLP-1 has been well defined.[17,18] Some amino acids, including H1 and W25, are important for GLP-1 to interact with its receptor,
and some others, including A2 and K28, are sensitive to enzyme digestion.\cite{17, 18} Enogenous enzymes, including dipeptidyl peptidase-4 and neutral endopeptidase, can rapidly inactivate GLP-1 by destroying its N-terminal and α-helix structures, leading to it having a short half-life of less than 5 min \textit{in vivo}.\cite{9} Based on the structure–activity relationship of GLP-1 we constructed mGLP-1 (A2G, K28R) and optimized its recombinant production in a previous study.\cite{16} These mutations are designed to enhance the potency of mGLP-1 \textit{in vivo}. Our previous study showed that mGLP-1 had a dose-dependent acute glucose-lowering effect in normal mice.\cite{16} The data from this study showed that the potency of mGLP-1 was greatly enhanced \textit{in vivo} after the site-directed mutation (Figure 2).

The mouse model of diabetes induced by multiple low doses of STZ is a well-established animal model for diabetes research and has been widely used.\cite{19} STZ specifically causes pancreatic tissue damage, impairs beta cell function and induces hyperglycaemia.\cite{19, 20} Compared with a single large dose of STZ, which is also used for induction of diabetes, multiple low doses of STZ more efficiently mimic the pathogenesis of diabetes and induce hyperglycaemia in mice.\cite{19, 20} Diabetes induced by this method is more like type 1 diabetes rather than type 2 diabetes in which insulin resistance plays a dominant role.\cite{19, 20} Since GLP-1 exerts its bioactivity through multiple pathways, including improving beta cell function, it can also be used for type 1 diabetes though it has mainly been developed for type 2 diabetes.\cite{5}
In mice with diabetes induced by STZ, mGLP-1 treatment greatly improved their glucose homeostasis (Figure 4). Our data clearly demonstrated mGLP-1 possessed a significant anti-diabetic effect in vivo. Moreover, compared with that of native GLP-1, the glucose-lowering activity of mGLP-1 was greatly improved, and this improvement may arise mainly from its enhanced potency in vivo.

The anti-diabetic effects of GLP-1 are mediated through multiple pathways. First, GLP-1 reduces food intake, thereby decreasing glucose absorption which mimics the effect of inhibitors of glycoside hydrolases. Second, GLP-1 stimulates insulin secretion, mimicking the effect of stimulators of insulin secretion. Third, GLP-1 alleviates insulin resistance in peripheral tissues, including the adipose tissue and muscle, mimicking the effect of sensitizers of insulin action. Additionally, GLP-1 increases beta cell mass in rodents, which is crucial for diabetes therapy. These multiple functions make GLP-1 an ideal candidate for anti-diabetic drug development. However, native GLP-1 has a poor potency in vivo, which limits its use in the clinical setting. As an analogue of GLP-1, mGLP-1 maintained its conformation and possessed an enhanced potency in vivo. Moreover, mGLP-1 also exerts its anti-diabetic effects through multiple pathways as demonstrated by its effects of reducing food intake, increasing insulin in the blood, as well as in the islets, and improving beta cell function. These multiple actions significantly enhanced the anti-diabetic effect of mGLP-1.

Figure 4 Treatment by mGLP-1 improved glucose homeostasis, elevated blood insulin and partially recovered pancreatic beta cell function in diabetic mice. (a) Glucose profiles after OGTT. (b) AUC of OGTT. (c) Blood insulin. (d) HOMA-β. Data are means ± SD, n = 6. *P < 0.05 vs saline-treated diabetic mice; **P < 0.01 vs saline-treated diabetic mice; #P < 0.05 vs GLP-1-treated diabetic mice.
In conclusion, in this study we clearly demonstrated that mGLP-1 possesses an improved glucose-lowering activity in diabetic mice. Our data indicate that this mutated peptide can be recognized as a potential candidate for the future development of anti-diabetic drugs.

**Declarations**

**Conflict of interest**

The Author(s) declare(s) that they have no conflicts of interest to disclose.

**References**

3. Eppens MC *et al*. Prevalence of diabetes complications in adolescents with

**Funding**

This work was supported by the China National Natural Science Foundation (81172974, 30973667), Jiangsu Province ‘Qing Lan Project’ (2010), and Jiangsu Province ‘333 High-level Talents Cultivation Project’.

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**Figure 5** Representative photographs of H&E staining and insulin IHC staining of tissue samples taken from diabetic mice. These photographs were taken by using an optical microscope and the magnification was set at 400 fold.
type 2 compared with type 1 diabetes. 


