Pro-GLP-1, a Pro-drug of GLP-1, is neuroprotective in cerebral ischemia

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ABSTRACT

Pro-Glucagon-like peptide-1 (Pro-GLP-1), a long-lasting GLP-1 receptor (GLP-1R) agonist, was developed using a polymeric pro-drug strategy and its neuroprotective effects on ischemic stroke were investigated in C57BL/6 mice. Pro-GLP-1 was injected into the intraperitoneal cavity of C57BL/6 mice once a day for 7 days before middle cerebral artery occlusion (MCAO) surgery. The neurological deficit score and TTC staining were determined 24 h after ischemia. The results demonstrated that Pro-GLP-1 was slowly degraded in the plasma and brain of the mice, and GLP-1 could be detected even 12 h after administration. Pro-GLP-1 was significantly neuroprotective in C57BL/6 mice subjected to MCAO. In cultured cortical neurons, treatment with Pro-GLP-1 attenuated apoptosis induced by oxygen–glucose deprivation (OGD). The neuroprotective effects of Pro-GLP-1 were blocked by a selective GLP-1 receptor antagonist and knockdown of GLP-1 receptor with shRNA. However, Pro-GLP-1 had no effect on blood glucose and insulin levels which indicated that neuroprotection was mediated by the activation of GLP-1 receptor in the brain. Pro-GLP-1 repaired the balance of pro- and anti-apoptotic proteins and decreased the expression of caspase-3. The anti-apoptotic effect was mediated by the cAMP/PKA and PI3 K/Akt pathway. Our research provides evidence that pre-treatment of MCAO mice with Pro-GLP-1 exerts a neuroprotective effect mediated by a blockade of apoptosis and that Pro-GLP-1 might be a potential neuroprotective agent candidate against ischemic stroke.

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1. Introduction

Ischemic stroke is a serious disease leading to death and disability as well as malignant tumors and heart disease which is induced by a reduction or even blockage of blood flow to the cerebrovascular system (Go et al., 2014). This pathological process is accompanied with a series of events including glutamate excitotoxicity, oxidative stress, nitric oxide production, calcium overload and inflammation (Tu et al., 2010; Lai et al., 2014). Multiple neuroprotective compounds have been reported to be effective against cerebral ischemia in animal and cell models (Gu et al., 2012; Mathai et al., 2012), but few of these drugs are clinically effective (Cook et al., 2012). Thrombolysis with intravenous tissue plasminogen activator (t-PA) is the only approved therapy of patients with acute ischemic stroke, but the narrow therapeutic window and side effects of t-PA have limited its clinical application (Yepes et al., 2009). Thus, new strategies and agents are urgent required for the treatment of ischemic stroke.

Glucagon-like peptide-1 (GLP-1) is an important gastrointestinal hormone which plays a crucial role in blood glucose control and pancreatic islet β cell proliferation (Sathananthan et al., 2013; Aulinger et al., 2014). GLP-1 is also a neuropeptide synthesized by neuronal cells, and GLP-1 receptor is widely expressed throughout the brain (Darsalia et al., 2013). GLP-1 receptor activation exerts a neuroprotective effect in cerebral ischemia (Li et al., 2009), modulates neuronal activity, protects against neuronal damage induced by various insults, and is involved in learning and memory (Greig et al., 2014; Seufert and Gallwitz, 2014). Therefore, GLP-1 receptors may serve as an effective therapeutic target for brain disorders. However, GLP-1 is rapidly degraded and inactivated shortly after secretion into the blood, which has limited its clinical use (Idorn et al., 2014). Thus, it is necessary to develop long-lasting GLP-1 analogues without the short-comings of native GLP-1, for possible clinical application (Doyle et al., 2003). GLP-1 binds the GLP-1 receptor via its NH2-terminal ectodomains (ECDs) (Castro et al., 2005; Hoare, 2005; Laburthe et al., 2007) and devel-
velopment of small molecule GLP-1 receptor agonists has been generally unsuccessful. Some agonists have been reported but they have low efficiency compared with GLP-1 (Sloop et al., 2010). Long-lasting peptide analogues such as liraglutide and exenatide were identified and investigated, and the neuroprotective efficiency of these analogs was also reported (Lee et al., 2011; Teramoto et al., 2011; Briyal et al., 2012; Darsalia et al., 2012; Darsalia et al., 2013; Sato et al., 2013; Yang et al., 2013; Holscher, 2014). However, these analogs have the potential to exert immunogenicity because their structure is different from native GLP-1 (Davies et al., 2011). Thus, new strategies should be applied in the development and identification of new long-lasting GLP-1 receptor agonists.

Using a polymeric pro-drug strategy, we have developed a new polymer of GLP-1 named Pro-GLP-1. The polymer was produced in *Escherichia coli* as a single polypeptide chain containing GLP-1 repeats. Pro-GLP-1 can be slowly digested by specific endoproteinase in vivo in which case bioactive GLP-1 is gradually released. Therefore, the bioavailability and bioactivity of GLP-1 is extended in vivo. In this study, we examined the neuroprotective effect of Pro-GLP-1 on cerebral infarction after the induction of middle cerebral artery occlusion (MCAO) in C57BL/6 mice.

2. Materials and methods

2.1. Release of GLP-1 from Pro-GLP-1

To demonstrate the in vivo release rate of GLP-1 from Pro-GLP-1, 1 nmol/kg of Pro-GLP-1 or 3 nmol/kg of GLP-1 was intraarterially injected into C57BL/6 mice. Blood samples and brain tissue from CA1 region of hippocampus were collected at different time points. The plasma samples were centrifugated at 2500 rpm for 5 min at 4 °C. Brain tissues were homogenized in PBS and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatants were used for further determination. The concentration of GLP-1 in the plasma and brain was measured by enzyme-linked immunosorbent assay (ELISA) using a GLP-1 ELISA kit (Millipore Corporation, Billerica, MA, USA).

Pro-GLP-1 at a final concentration of 100 μM was incubated with 20 μl of human plasma at 37 °C for 12 h. The reaction mixture (50 μl) was sampled at the indicated time points. Sodium dodecyl sulphate (SDS) loading buffer was added to the samples to terminate the enzymatic reaction. Western blot analysis was performed to determine the level of GLP-1 at different time points.

2.2. Detection of exendin-4 Level in the brain and plasma

Determination of exendin-4 levels in the brain and plasma are described as follows. Mice were intraperitoneally injected with 1 nmol/kg exendin-4. Brain tissue and plasma samples were collected at different time points after administration. Exendin-4 levels in the brain and plasma were determined by EIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). Plates were read at a wavelength of 450 nm on a SpectraMax 190 enzyme-linked immunosorbent assay plate reader.

2.3. Determination of blood glucose and plasma insulin levels

Pro-GLP-1 (0.3, 1.0 and 3.0 nmol/kg) or saline were intraperitoneally injected into C57BL/6 mice, and blood glucose and plasma insulin levels were measured 0 and 30 min after administration. The blood glucose levels were measured at the indicated time using a glucometer (Roche Diagnostics, Mannheim, Germany). Plasma insulin levels were determined by ELISA kit (Millipore). The optical density was monitored at a wavelength of 490 nm.

Detection values were converted using a standard curve performed in parallel.

2.4. Experimental animals

Male C57BL/6 mice weighting between 18 and 22 g were used in our study. All animal protocols were approved by the Animal Care and Use Committee of Fourth Military Medical University and every effort was made to minimize the number of animals used and minimize their discomfort. Mice were randomly divided into 5 groups (8 animals per group). Group 1, Sham group (Saline), Group 2, MCAO + Vehicle (Saline), Group 3, MCAO + 0.3 nmol/kg Pro-GLP-1, Group 4, MCAO + 1 nmol/kg Pro-GLP-1, Group 5, MCAO + 3 nmol/kg Pro-GLP-1. Saline or Pro-GLP-1 was administered by intraperitoneal injection once-a-day for 7 consecutive days. Mice were subjected to MCAO surgery after the last administration of Pro-GLP-1. Neurological deficits, volume of the infarcted lesions and nissl staining were measured 24 h after ischemia.

2.5. The middle cerebral artery occlusion (MCAO) Model

The MCAO model was performed according to a previously published protocol (de la Rosa et al., 2014). Briefly, mice were anesthetized with 1% pentobarbital sodium. The external carotid artery (ECA) and the right common carotid artery (CCA) were isolated. The incision near the ECA–CCA branch was inserted into a nylon filament coated with silicon resin and advanced about 9–11 mm. Reperfusion was performed 90 min after MCAO. The procedure performed in the sham group was the same as the model group but the nylon filament was advanced only about 5 mm. The body temperature of each animal was held at 37 °C during surgery until recovery from anesthesia after surgery.

2.6. Evaluation of neurological deficits

Neurological deficit scores were analyzed according to Bederson’s score (Dong et al., 2013), and was defined as follows: Grade 0, no deficit observed; Grade 1, flexion to contralateral torso and forelimb; Grade 2, decreased resistance to lateral push but without circling; Grade 3, leaning to affected side; and Grade 4, no spontaneous locomotor activity. Higher neurological deficit scores indicated more severe impairment of motor injury.

2.7. 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining

After the evaluation of neurological deficit scores, mice were killed and their brains were removed at 4 °C, then frozen at −20 °C for 1 h. TTC staining was performed to analyze the infarct volume. Brain tissue was sliced into 1 mm sections, the slices were incubated in 1.5% TTC solution for 30 min at 37 °C, and fixed in 4% paraformaldehyde for 24 h at room temperature. The tissue slices were digitally scanned and the volume of the infarction was analyzed using photo shop CS4 software.

2.8. Nissl staining

Mice were killed and their brains were perfused with saline followed by 4% paraformaldehyde. The prefrontal cortex and dorsal hippocampal area section were selected for nissl staining. Briefly, each brain section (20 μm) was cut using a frozen slicer (Leica Microsystems) and incubated in chloroform and acetone for 15 min, then switched to alcohol at a gradient concentration of 100%, 95% and 70% for 1 min. The slices were washed 3 times and incubated with 1% cresyl violet (containing 0.25% glacial acetic acid) for 20 min.
2.9. Intracerebroventricular Infusion of small hairpin RNA (shRNA)

Expression of GLP-1 receptor in the CA1 region of hippocampus was knocked down by intracerebroventricularly injecting shRNA. The specific sequence of shRNA targeting to GLP-1R (GenBank Accession No. NM_021332.2, nucleotides 608–628) mRNA sequence were constructed into the pGV118 lentiviral vector (GeneChem Co., Ltd., Shanghai, China). High purity (>1.9 ratio 260/280 Abs) transfection ready vector DNA was prepared and resuspended in TE (pH 8.0). The shRNA target sequence was GCCCTCAAGTGGATGTATAGC (5′–3′ with the titre of 10^6 TU/ml). A scrambled shRNA sequence TTCTCCGAACGTGTCACGT (5′–3′ with the titre of 10^6 TU/ml) was chosen for negative control. The lentiviral was dissolved in Enhanced Infection Solution (GeneChem Co., Ltd.) and administrated intracerebroventricularly by the volume of 1 μl per mouse. Briefly, mice were anesthetized with 1% pentobarbital sodium, and then placed in a mouse stereotaxic instrument. Negative control or lentivirus containing shRNA complimentary to GLP-1 receptor was stereotaxically injected into the right lateral ventricle (bregma: −0.58 mm; dorsoventral: 2.1 mm; lateral: 1.2 mm) of each mouse. After injection, the needle stayed in the brain for 5 min and was withdrawn at the rate of 1 mm/min. Mice were returned to their cages after they recovered from the anesthesia.

2.10. Primary mouse cortical neuronal culture and oxygen-glucose deprivation (OGD)

Primary mouse cortical neurons were cultured from embryonic 18 day-old C57BL/6 mice. After the brain was removed from the embryo, the prefrontal cortex was carefully dissected, minced, and trypsinized using 0.125% trypsin. Neurons were seeded into 96 or 6 well plates with DMEM containing 20% fetal bovine serum, the density of the neuron was about 2500 cells/mm^2. Twenty four hours later, the culture medium was replaced with neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 2% B27 (Invitrogen) and 2 mm glutamine (Invitrogen). These cultures were maintained in 95% air 5% CO_2 with 95% humidity at 37 °C. Neurons were used for further analysis 7 days after culture. OGD was performed as previously reported (Meloni et al., 2001), briefly, neurons were washed three times with PBS, placed in glucose-free EBSS buffer and incubated for 6 h in an anoxic chamber which was filled with 95% N_2 and 5% CO_2 at 37 °C. Neurons were then returned to normoxic conditions for reoxygenation and the culture medium was replaced by fresh neuron-based media for 12 h.

2.11. cAMP accumulation assay

Neurons cultured for 7 days were incubated with GLP-1, exendin-4 or Pro-GLP-1 at different concentrations for 30 min at 37 °C, then digested with trypsin and centrifuged at 800 rpm for 10 min at 4 °C. The supernatant was discarded, the neurons were resuspended in cell lysis buffer, and the cells were thawed by gentle mixing. The cellular debris was removed by centrifugation and the supernatants were lyophilized. cAMP was measured using the cAMP parameter assay kit (R&D, Minneapolis, MN, USA). All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently normalized to the response of 1 mm GLP-1. Triplicate plates were analyzed for each data point.

2.12. MTT and lactate dehydrogenase (LDH) assay

Cell viability was measured by the MTT and LDH assay. Cells were seeded into 96-well plates with 100 μl neurobasal medium per well. After the preprocessing, the cells were treated with different concentrations of Pro-GLP-1 (0, 2.5, 10, and 40 nm) 1 h before OGD. After OGD and reperfusion for 12 h, MTT (0.5 mg/ml) was added to each well and then incubated at 37 °C for 4 h. The culture medium was replaced with dimethyl sulfoxide (DMSO) and the absorbance at 490 nm was measured using a microplate reader. Cell injury was assessed by measuring the LDH released using a cytotoxicity detection kit according to the manufacturer’s instructions (JianCheng Co, Nanjing, China). The absorbance was measured at 490 nm. The total LDH release was determined in the neurons treated with 1% Triton X-100 for 24 h. Cells maintained under normoxic conditions were defined as 100% viable, and the viability of cells exposed to OGD conditions is expressed as a percentage compared to neurons under normoxic conditions. Triplicate plates were analyzed for each data point.

2.13. Hoechst/propidium iodide (PI) double staining

Twenty-four hours after OGD/R conditions, neurons were stained with 10 μg/ml Hoechst 33,258 (Sigma, Saint Louis, MO, USA) and 10 μg/ml PI (Sigma). After staining for 15 min, the cells were fixed with formaldehyde for 20 min and observed under a fluorescence microscope (Olympus BX61, Japan). The Hoechst and PI were excited at 340 nm and 620 nm respectively. Three visual fields were randomly selected in each sample.

2.14. Cell treatment paradigm

Pro-GLP-1 was dissolved in ddH_2O and added to medium at a final concentration of 10 nm 1 h before OGD. PKA, P3 K and MEK inhibitors were purchased from selleck Chemicals (Houston, TX, USA). H89 (10 μM, Selleck, no. S1582), wortmannin (10 μM, Selleck, no. S2758) and U0126 (10 μM, Selleck, no. S1102) were added to the cultures 2 h before OGD injury and remained in the culture throughout Pro-GLP-1 treatment. Neurons in the OGD group were treated with an equal volume of ddH_2O, while the normoxia group did not receive any treatment.

2.15. Western blot

Protein sample (10 μg) was loaded and subjected to SDS–PAGE at 200 V for 45 min and then transferred to polyvinylidene fluoride membranes at 100 V for 2 h. The membranes were incubated with anti-pAKT antibody (1:1000; CST, Danvers, MA, USA), anti-p-CREB (1:1000; CST), anti-Bax (1:500; Santa Cruz Biotechnology, Dallas, Texas, USA), anti-BCL-2 (1:500; Santa), anti-Caspase-3 (1:500; Santa Cruz) and anti-β-actin (1:5000; Sigma, Saint Louis, MO, USA) at 4 °C overnight. Goat anti-mouse or anti-rabbit IgG secondary antibody (Santa Cruz) was diluted 1:10,000 and the membranes were incubated with this antibody concentration for 1 h. Then the bands on the film were scanned and analyzed.

2.16. Statistical analysis

Data were presented as means ± SEM. SPSS13.0 was used to perform statistical analysis. The differences between groups were analyzed with one-way ANOVA followed by Dunnert T-test. A p < 0.05 was considered to be significant.

3. Results

3.1. Pro-GLP-1 continuously released GLP-1

The schematic diagram of Pro-GLP-1 was shown in Fig. 1A. To determine the release of GLP-1 from Pro-GLP-1 in vitro, Pro-GLP-1 was incubated with human plasma. Western blot analysis was
used to determine the amount of GLP-1 released over time. After incubation for 15 min, soluble GLP-1 was detectable in the plasma, and this amount progressively increased during the 12 h of incubation (Fig. 1B). These results indicated that GLP-1 was continuously released from Pro-GLP-1 for at least 12 h.

To detect the release of GLP-1 in vivo, a single dose of Pro-GLP-1 or GLP-1 was intraperitoneally administered to C57BL/6 mice. GLP-1 was metabolized rapidly in vivo which was hardly detectable 50 min after injection. However, compared with the short half-life of GLP-1, Pro-GLP-1 was slowly degraded in the plasma, and GLP-1 could be detected even 12 h after administration (Fig. 2A). We next determined the amount of GLP-1 in the brain following GLP-1 or Pro-GLP-1 injection. Native GLP-1 was barely detectable in the brain after injection because it was quickly metabolized in the plasma. However, in the mice that had been injected with Pro-GLP-1, GLP-1 was detected in the brain 12 min after intraperitoneal administration, and this amount gradually increased over time. GLP-1 was still detected 12 h after administration of Pro-GLP-1 (Fig. 2B). These results indicated that Pro-GLP-1 was gradually degraded in the tissues after administration and this process continuously released GLP-1.

3.2. Pro-GLP-1 activated GLP-1 receptor signaling with high efficacy

GLP-1 ligation of the GLP-1 receptor activates changes in cyclic adenosine monophosphate (cAMP) concentration (Van de Velde et al., 2011). Therefore, GLP-1 receptor signaling induced by Pro-GLP-1 was evaluated by detecting changes in cAMP concentration in primary cultured neurons. Pro-GLP-1 had an EC50 of 2.3 nm with 100% potency relative to maximum stimulation by GLP-1 in primary cultured neurons, while exendin-4 had an EC50 of 0.15 μM and only 82% potency compared with maximum stimulation by GLP-1 (Fig. 3A). Treatment with exendin (9–39), a GLP-1 receptor antagonist, blunted the GLP-1 receptor signaling activated by Pro-GLP-1 (Fig. 3B). These results indicated that Pro-GLP-1 stimulated the GLP-1 pathway with high efficacy and low EC50.

3.3. Pro-GLP-1 preconditioning improved functional outcome and reduced infarction size caused by MCAO

The neuroprotective effect of Pro-GLP-1 was evaluated in MCAO mice. The MCAO injury lead to a higher deficit score in mice at 24 h

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**Fig. 1.** Pro-GLP-1 continually released native GLP-1. (A) The structure of Pro-GLP-1 and the cleavage site. Pro-GLP-1 was metabolized by specific enzymes in vivo and continually released bioactive GLP-1. (B) Represent western blot showing the degradation of Pro-GLP-1 into GLP-1.

**Fig. 2.** GLP-1 was continually detected in the plasma and brain after intraperitoneal administration of Pro-GLP-1. (A) The level of GLP-1 in the plasma of C57BL/6 mice after a single intraperitoneal injection of 1 nmol/kg Pro-GLP-1 or 4 nmol/kg GLP-1. (B) The level of GLP-1 in the brain of C57BL/6 mice after a single intraperitoneal injection of 1 nmol/kg Pro-GLP-1 or 4 nmol/kg GLP-1.

**Fig. 3.** Pro-GLP-1 activated GLP-1 receptor signaling. (A) Dose–effect curve of Pro-GLP-1, GLP-1 and exendin-4 on the intracellular concentration of cAMP in neurons. (B) Effect of exendin (9–39) on Pro-GLP-1-induced signaling.
after reperfusion compared with the sham group (Fig. 4A). However, mice pre-treated with all three dose of Pro-GLP-1 demonstrated a significant decrease in neurological deficit scores in a dose-dependent manner compared with the vehicle group. The TTC staining results indicated that MCAO injury produced an infarction area that spanned the right frontal, parietal, and occipital cerebral cortices. The infarct volume was significantly reduced in the Pro-GLP-1 preconditioned group in a dose-dependent manner compared with the control groups (Fig. 4B and C).

### 3.4. Pro-GLP-1 preconditioning increased neurons survival against OGD

The cerebral ischemia and reperfusion injury can be mimicked in vitro by OGD and reperfusion condition. To evaluate the effect of Pro-GLP-1 on OGD, neuronal cell viability was determined using the MTT and LDH assays. These analyses showed that neuronal survival was decreased after exposure to OGD. However, when the neurons were pretreated with Pro-GLP-1, cell survival was significantly increased compared with the OGD group and this neuroprotective effect was abrogated by GLP-1 receptor antagonist exendin (9–39) (Fig. 5A and B).

To further evaluate the neuroprotective effect of Pro-GLP-1, the rate of apoptosis was further evaluated using Hoechst 33,258 and PI double staining. The results showed that OGD caused a significantly higher rate of cell apoptosis compared with the normoxia group. All three concentrations of Pro-GLP-1 decreased the percentage of dead cells caused by OGD. However, this trend was diminished by the GLP-1 receptor antagonist exendin (9–39) (Fig. 5C and D).

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**Fig. 4.** Pro-GLP-1 preconditioning improved functional outcome and reduced infarction volume caused by MCAO. (A) Representative neurological deficit score 24 h after MCAO. (B) TTC staining results of brain slices 24 h after MCAO. (C) Quantitative analysis of B, data are shown as means ± SEM (n = 6 for each group). ## p < 0.01 compared with Sham group, * p < 0.05 compared with MCAO + vehicle group, ** p < 0.01 compared with MCAO + vehicle group.

### 3.5. The neuroprotective effect of Pro-GLP-1 was dependent upon activation of GLP-1 receptor

To further determine whether the neuroprotective effect of Pro-GLP-1 was dependent on the expression of the GLP-1 receptor, the shRNA lentiviral vectors were used to suppress GLP-1 receptor expression in the CA1 region of the hippocampus. The shRNA lentiviral constructs were applied to the brain region of each C57BL/6 mouse by stereotaxis injection, and parallel experiments were applied using a negative control shRNA. Seven days after stereotaxis injection of the shRNA, there was no significant difference in GLP-1 receptor gene or protein expression between the mice in the control (untreated with shRNA) group and the mice that had been injected with negative shRNA. However, GLP-1 receptor expression was significantly reduced to 30% in the CA1 region 7 days after the unilateral administration of the shRNA vector targeting GLP-1 receptor. This inhibition lasted for at least 21 days after the injection, both at the gene and protein level (Fig. 6A–C). Neurological deficit evaluation and TTC staining results showed that, the negative shRNA had no effect on the protective effect of Pro-GLP-1, but GLP-1 receptor shRNA almost completely blocked the beneficial effect of Pro-GLP-1 administration (Fig. 6D–F).

### 3.6. Pro-GLP-1 mediated anti-apoptotic effect against MCAO injury

Apoptosis is one of the major causes of neuronal death after cerebral ischemia (Zhao et al., 2014). Bcl-2 and Bax represent the anti-apoptotic and pro-apoptotic proteins of the Bcl family and the ratio between Bax and Bcl-2 can be an indicator of susceptibility to cerebral ischemia injury (Hasegawa et al., 2010). The Bax and Bcl-2 level in peri-infarct tissue was detected with western blot analysis. This analysis showed that the ratio between Bax and Bcl-2 was significantly increased 4 times 24 h after cerebral ischemia. This ratio increased because Bax was upregulated and Bcl-2 was down-regulated. This trend was significantly reversed by Pro-GLP-1 preconditioning, while the administration of GLP-1 receptor shRNA diminished this effect (Fig. 7A). The level of active caspase-3 is an indicator of apoptosis (Liang et al., 2014). Western blot analysis showed that MCAO lead to a significant increase in active caspase-3 in the brain compared with the control group, while Pro-GLP-1 preconditioning reversed this trend and GLP-1 receptor shRNA administration diminished this trend (Fig. 7B).

Nissl staining results showed that cerebral ischemia and reperfusion injury led to neuronal damage, both in the prefrontal cortex and hippocampus CA1 region. The injured neurons presented as shrunken cell bodies accompanied by shrunken and pyknotic nuclei. Pro-GLP-1 preconditioning significantly decreased the number of injured neurons following MCAO injury. This effect was blocked by GLP-1 receptor shRNA administration (Fig. 7C–E). These results showed that Pro-GLP-1 mediated a neuroprotective effect against MCAO by inhibiting apoptosis.
3.7. The anti-apoptotic effect of Pro-GLP-1 was mediated by the cAMP/PKA and PI3K/Akt pathway

The downstream activation of GLP-1 receptor leads to the activation of signaling pathways such as cAMP/PKA and PI3K/Akt which play an important role in the process of growth, apoptosis and proliferation of neurons (Hui et al., 2003). The stimulation of these signaling pathways is dependent upon cell type, environmental conditions and cellular stimuli (Wang et al., 2012). Pro-GLP-1 decreased the amount of cell death caused by OGD,
and this beneficial effect was blocked by wortmannin (an inhibitor of PI3 K) and H89 (an inhibitor of PKA). However, the ERK inhibitor, U0126, had no impact on the effect of Pro-GLP-1 (Fig. 8A and B). Western blot analysis indicated that the protein level of Bax and active caspase-3 was upregulated while Bcl-2 was down regulated, and the ratio of Bax/Bcl-2 was increased after OGD compared with the normoxia group. This observation is in accordance with the in vivo data. Pro-GLP-1 reversed this trend since the neuroprotective effect of Pro-GLP-1 was blocked by the PI3 K inhibitor wortmannin and the PKA inhibitor H89 but not by the ERK inhibitor U0126 (Fig. 8C and D). These results indicated that Pro-GLP-1 conferred a neuroprotective effect against cerebral ischemia via the cAMP/PKA and PI3 K/Akt pathway but this effect was independent of the ERK pathway.

4. Discussion

Since the GLP-1 receptor is expressed in the brain, it is reasonable to assume that GLP-1 may be neuroprotective in neurological diseases. Perry et al. reported that GLP-1 had a beneficial effect in excitotoxic neuronal damage (Perry et al., 2002) and the protective effect of GLP-1 in cerebral ischemia has also been reported.
However, the short half-life of GLP-1 has limited its clinical application. Thus, long-lasting GLP-1 analogs have been investigated for their neuroprotective activity in these neurological pathologies.

GLP-1 receptor is a member of the 7 transmembrane G-protein coupled receptor family. GLP-1 binds GLP-1 receptors via a two-step mechanism (Castro et al., 2005; Hoare, 2005; Laburthe et al., 2007). The initial binding step is the interaction between the COOH-terminal amino acids of GLP-1 and the ECD of the GLP-1 receptor. The second phase of receptor activation is occurs when the NH2-terminal of GLP-1 interacts with the transmembrane domains of the GLP-1 receptor to induce the active conformation. Traditional small molecules are unable to form this unique structural architecture and therefore cannot modulate the activity of the GLP-1 receptor. Thus, there has been significant difficulty in identifying low molecular weight organic and non-peptide molecules that can bind the GLP-1 receptor and few candidate molecules have been reported. The design of long-lasting GLP-1 receptor agonists has been limited to 30–40 amino acid peptides. Exendin-4 is the most commonly used long-lasting GLP-1 receptor agonist and it has about 50% homology to native GLP-1. However, the altered structure of exendin-4 reduces its affinity for the GLP-1 receptor and our data has shown that the EC50 of exendin-4 was almost 10-fold higher than that of native GLP-1. Pro-GLP-1 activates the GLP-1 receptor by gradual degradation and release of soluble GLP-1. Thus, the EC50 of Pro-GLP-1 was comparable to that of native GLP-1. Pro-GLP-1 continually releases GLP-1 for 12 h, while exendin-4 reached its optimal potency after only 1 h after administration and it was hardly detected 4 h after intraperitoneal injection in the plasma and the brain (Supplementary Fig. 2). Our results are in accordance with previous reports (Hadjiyanni et al., 2008; Gao and Jusko, 2011; Li et al., 2012). Thus, Pro-GLP-1 is a pro-drug of GLP-1 with a long half-life and low EC50.

Previous reports have shown that activation of the GLP-1 receptor resulted in an improvement of glycemic control, which could lower blood glucose and stimulate insulin release (Skoglund et al., 2000; Holst and Gromada, 2004). Hyperglycemia is one of the major independent risk factors for cerebral ischemia (Kamada et al., 2007). Insulin was found to confer a neuroprotective effect in cerebral ischemia in MCAO (Sanderson et al., 2009). The GLP-1 analog exendin-4 was reported to confer a profound neuroprotective effect in a MCAO model of diabetic rats (Darsalia et al., 2012). Thus, a peripheral effect on hyperglycemia induced by the GLP-1 receptor agonist is necessary to be excluded in non-diabetic mice. Since the concentration of native GLP-1 was continually detected in the brain of our Pro-GLP-1-treated mice, and knock-down of the GLP-1 receptor in the hippocampus blocked the neuroprotective effect of Pro-GLP-1, we can conclude that Pro-GLP-1 conferred a neuroprotective effect on cerebral ischemia in this model by activating the GLP-1 receptor in the brain. Taken together, we
conclude that pro-GLP-1 is a long-lasting GLP-1 receptor agonist and has the potential for the treatment of cerebral ischemia.

Conflict of interest
The authors declare no conflict of interest.

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Appendix A. Supplementary material
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2015.01.010.

References


