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Exendin-4 attenuates neuronal death via GLP-1R/PI3K/Akt pathway in early brain injury after subarachnoid hemorrhage in rats

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Abstract

Neuronal apoptosis is considered to be a crucial therapeutic target against early brain injury (EBI) after subarachnoid hemorrhage (SAH). Emerging evidence indicates that Exendin-4 (Ex-4), a glucagon-like peptide 1 receptor (GLP-1R) agonist, plays a neuroprotective role in cerebrovascular disease. This study was conducted in order to verify the neuroprotective role of EX-4 in EBI after SAH in rats. The endovascular perforation model of SAH was performed in Sprague-Dawley rats (n=153). Ex-4 was intraperitoneally injected 1 hour after SAH induction in the rats (SAH+Ex-4). To elucidate the underlying molecular mechanism, small interfering ribonucleic acid (siRNA) for GLP-1R and a specific inhibitor of PI3K, LY294002, were injected intracerebroventricularly into SAH+Ex-4 rats before induction of SAH (n=6 per group). SAH grading evaluation, immunohistochemistry, Western blots, neurobehavioral assessment, and Fluoro-Jade C (FJC) staining experiments were performed. Expression of GLP-1R was significantly increased and mainly expressed in neurons at 24 hours after SAH induction. Administration of Ex-4 significantly improved both short- and long- term neurobehavior in SAH+Ex-4 group compared to SAH +Vehicle group after SAH. Ex-4 treatment significantly increased the expression of GLP-1R, PI3K, p-Akt, Bcl-xl, and Bcl-2, while at the same time was found to decrease expression of Bax in the brain. Effects of Ex-4 were reversed by the intervention of GLP-1R siRNA and LY294002 in SAH+Ex-4+GLP-1R siRNA and SAH+Ex-4+LY294002 groups, respectively. In conclusion, the neuroprotective effect of Ex-4 in EBI after SAH was mediated by attenuation of neuronal

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Conflict of interest

The authors declare no conflict of interest.

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apoptosis via GLP-1R/PI3K/Akt signaling pathway, therefore EX-4 should be further investigated as a potential therapeutic agent in stroke patients.

Keywords

Subarachnoid hemorrhage; Exendin-4; GLP-1R; Neuronal apoptosis; PI3K/Akt signaling

1. Introduction

Subarachnoid hemorrhage (SAH) is a devastating subtype of stroke that is associated with a 40–50% 30-day mortality rate, as well as leaving 8–20% of its victims in a permanently disabled state (Nieuwkamp et al., 2009; van Gijn et al., 2007). In recent years, early brain injury (EBI) has been reported as a primary predictor of long-term neurological deficits after SAH (Fujii et al., 2013; Sehba et al., 2012). Neuronal apoptosis, which is recognized as one of the most crucial pathophysiological processes underlying EBI, is considered to be a potential therapeutic target against SAH (Chen et al., 2014).

Glucagon-like peptide 1 (GLP-1) is a gut hormone released from intestinal L cells in response to oral glucose intake (Ahren, 1998). When GLP-1 exerts its effects alone without involvement of glucose-dependent insulinotropic polypeptide (GIP), it can serve as an incretin factor which acts to reduce blood glucose levels by stimulating insulin secretion and suppressing glucagon release (Yamato et al., 1990). It soon became a favorable therapeutic target to treat type 2 diabetes mellitus when researchers found that its stimulation of insulin was in a glucose-dependent manner (Parhofer, 2007). An interesting fact is that GLP-1 does not stimulate insulin when plasma glucose concentration is in normal fasting range, which reduces the risk of hypoglycemia as the most feared complication of anti-diabetic agents (Rask et al., 2004). However, an exceptionally short half-life (<2 minutes) of endogenous GLP-1 has been reported due to its rapid cleavage via dipeptidyl peptide 4 (DDP-4) enzyme (Kieffer et al., 1995). As a result, Exendin-4 (Ex-4), an analogue of GLP-1 with prolonged half-life time (2.4 hours) (Egan et al., 2002; Li et al., 2011) was synthesized to treat adult type 2 diabetes, which was approved by FDA in 2005 (Briones and Bajaj, 2006).

There is emerging evidence that besides its anti-diabetic effects, Ex-4 has the potential to carry out a neuroprotective role in central nervous system (CNS) disease (Harkavyi and Whitton, 2010; Li et al., 2009; Perry and Greig, 2005). It has been proven that Ex-4 exerts its protection by specifically binding to glucagon-like peptide 1 receptors (GLP-1R) (Mann et al., 2007) which are widely expressed in CNS, especially on neurons (Alvarez et al., 1996; Gu et al., 2013). It has also been reported that GLP-1R agonists participate in neuroprotective action against ischemic stroke by reducing apoptosis via phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (Zhu et al., 2016). However, the potential neuroprotective benefits of Ex-4 have not been illustrated in SAH. The present study aimed to assess the effects of Ex-4 on neurobehavioral outcome and neuronal apoptosis after SAH in rats. The mechanisms of those potential effects were explored using GLP-1R siRNA and LY294002.

2. Materials and Methods

2.1. Animals

All experimental procedures were performed following a corresponding study protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University in accordance with the NIH Guide for the Care and Use of Laboratory Animals. A total of 153 adult male Sprague-Dawley rats (280–350g, Harlan, Indianapolis, IN, USA) housed in a light and temperature controlled room with sufficient food and water were used in this study.

2.2. SAH model and study design

The endovascular perforation model of SAH was performed following previously described procedures (Duris et al., 2011; Zhan et al., 2012). Briefly, the rats were intubated in deep anesthesia and anesthesia was maintained with 3% isoflurane in 70/30% medical air/oxygen by a rodent ventilator (Harvard Apparatus, Holliston, MA, USA). A sharpened 4-0 nylon suture was introduced into the left internal carotid artery through a dissociated external carotid artery stump until resistance was detected. The suture was advanced 3 mm further to perforate the bifurcation of the anterior and middle cerebral artery followed by immediate withdrawal. In sham-operated animals, the same procedure were performed before the puncture when suture was retracted once resistance was felt.

All rats were randomly assigned to the following experiments as described (Fig. 1).

Experiment 1—To determine the time course of endogenous GLP-1R expression after SAH by Western blot, 39 rats were divided into six groups: Sham (n=6), SAH 3 hours (n=6), SAH 6 hours (n=6), SAH 12 hours (n=6), SAH 24 hours (n=9), SAH 72 hours (n=6). Double immunohistochemistry staining of GLP-1R with neurons, astrocytes and microglia was performed at 24 hours after SAH (n=3).

Experiment 2—To evaluate the short-term neurobehavioral function and neuronal apoptosis, 39 rats were assigned to five groups: Sham (n=9), SAH+Vehicle (1 mL sterile 0.9 % of NaCl, n=9), SAH+Ex-4 (1 µg) (1 µg in 1 mL sterile 0.9 % of NaCl, n=6, Sigma-Aldrich, St. Louis, MO, USA), SAH +Ex-4 (3 µg) (3 µg in 1 mL sterile 0.9 % of NaCl, n=9) (Kuroki et al., 2016; Lee et al., 2011), SAH+Ex-4 (9 µg) (9 µg in 1 mL sterile 0.9 % of NaCl, n=6). Vehicle or Ex-4 was intraperitoneally (i.p) delivered at 1 hour after SAH. SAH grading score, modified Garcia test and beam balance test were assessed at 24 hours after SAH (n=6 per group). Blood glucose level was tested at 8 time points during the first 24 hours (n=6 per group) after SAH. The left hemisphere samples from rats in those five groups were collected at 24 hours after SAH for further Western blots (n=6 per group). After choosing the best dosage, Fluoro-Jade C (FJC) staining was conducted with the samples from Sham, SAH+Vehicle, and SAH+Ex-4 (3µg) group (n=3 per group). Based on results of Experiment 2, Ex-4 (3 µg) was chosen for future experiments.

Experiment 3—To assess the long-term neurobehavioral function, 24 rats were divided into three groups: Sham, SAH+Vehicle, SAH+Ex-4 (n=8 per group). The rotarod test was

performed on day 7, 14, and 21 after SAH while Morris water maze was conducted on days 21–25 after SAH.

Experiment 4—24 rats were randomly assigned to 4 groups for mechanism study: SAH +Ex-4+DMSO (5 μ L of 25% dimethyl sulfoxide in phosphate-buffered saline (PBS), SAH +Ex-4+LY294002 (50 mmol/L in 25% DMSO of PBS) (Endo et al., 2006; Hao et al., 2014), SAH+Ex-4+Scr siRNA (500 pmol of scrambled siRNA) (He et al., 2015), SAH +Ex-4+GLP-1R siRNA (500 pmol of GLP-1R siRNA) (He et al., 2015) (n=6 per group). DMSO and LY294002 were intracerebroventricularly (i.c.v) delivered 30 minutes before SAH (Endo et al., 2006) while scrambled siRNA and GLP-1R siRNA were injected intracerebroventricularly 48 hours before SAH (Salahpour et al., 2007). Western blots were performed with the left hemisphere samples collected from all groups, including Experiment 2 at 24 hours after SAH (n=6 per group).

2.3. Blood glucose level

Plasma glucose level was measured before SAH, at 0 (immediately), 30, 60, 90, 120, 180 minutes and 24 hours after delivering vehicle or drug (Kuroki et al., 2016). Those measurements were performed by a blood glucose meter (Ascensia Diabetes Care US Inc., NJ, USA) using the blood collected from the tail vein.

2.4. SAH grade

The base of rat brain was photographed immediately after euthanasia and assessed by an independent observer as previously described (Sugawara et al., 2008). Briefly, the total score (maximum SAH grade=18) was calculated as the sum of six sub-scores based on six corresponding predetermined areas. Animals with mild SAH (SAH grade ≤ 7) were excluded according to the previous study (Sugawara et al., 2008).

2.5. Short-term neurological evaluation

Short-term neurobehavioral deficits were blindly assessed with the modified Garcia test and the beam balance test at 24 hours after SAH as previously described (Garcia et al., 1995; Suzuki et al., 2010). Modified Garcia test (maximum score=18) consists of six subtests, which include spontaneous activity, spontaneous movement of four limbs, forelimbs outstretching, vibrissa touch, body proprioception, and climbing capacity. The beam balance test was performed to evaluate the ability of animals to walk on a narrow wooden beam for 1 minute. The mean score was calculated based on three consecutive trials scored from 0 to 4 according to the walking ability.

2.6. Long-term neurobehavioral evaluation

In the long-term neurobehavioral study, the rotarod test was performed to assess sensorimotor coordination and balance while Morris water maze was chosen to evaluate spatial learning capacity, and reference memory as previously described (Lekic et al., 2010; Sherchan et al., 2011).

Briefly, rotarod (Columbus Instruments, Columbus, OH) consists of a rotating horizontal cylinder (7 cm diameter) that is divided into 9.5-cm-wide lanes. Animals had to keep

walking forward after being placed on the cylinder. The cylinder started at 5 revolutions per minute (RPM) and 10 RPM, respectively, and accelerated by 2 RPM every 5 seconds. Latency to fall off was recorded by a photobeam circuit. The basic procedures of Morris water maze was performed according to a well-recognized protocol (Vorhees and Williams, 2006). The water maze is a circular metal tank, the inside of which is painted black and filled with water. A platform was placed at the center of one of the quadrants. In this test, animals were required to find the submerged platform, which was recorded by an overhead camera. In the test of spatial learning memory, animals were placed using a semi-random set of start locations. The time limits for each animal to find the platform was 60 seconds. After that, the animal was lead to the platform where it stayed for 5 seconds. The probe trial was performed at the last day of water maze test in which animals were allowed to search the preexistent platform area after removing the actual platform. Swim path, escape latency, and swim distance were recorded by a computerized tracking system (Noldus Ethovision; Noldus, Tacoma, WA, USA).

2.7. Intracerebroventricular administration

Intracerebroventricular (i.c.v) drug administration was performed as previously described (He et al., 2015). Rats were placed in a stereotaxic apparatus under anesthesia with 2.5% isoflurane in 70/30 % medical air/oxygen. The needle of a 10 μ L Hamilton syringe (Microliter701; Hamilton Company, Reno, NV, USA) was inserted into the right lateral ventricle through a burr hole using the following coordinates relative to bregma: 1.5 mm posterior, 0.9 mm lateral, and 3.3 mm below the horizontal plane of the bregma. Drugs and siRNA were infused directly into the lateral ventricles at a rate of 1 μ L/min by a pump. The needle was retracted 5 minutes after the injection when the burr hole was plugged with bone wax immediately. LY294002 (Selleck Chemicals, Houston, USA) was prepared at 50 mmol/L in PBS (contains 25% DMSO), and 5 μ L of the DMSO and LY294002 were infused 30 minutes before SAH induction in the SAH+Ex-4+DMSO and SAH+Ex-4+LY294002 rats, respectively. GLP-1R siRNA and scrambled RNA (OriGene Technologies, Rockville, MD, USA) were prepared at 500 pmol in RNase free suspension buffer and were infused (5 μ L of the siRNAs) 48 hours before SAH modeling in the SAH+Ex-4+GLP-1R siRNA and SAH+Ex-4+Scr siRNA rats, respectively.

2.8. Immunohistochemistry staining

The brain samples for immunohistochemistry staining were prepared following previously reported procedures (Enkhjargal et al., 2016). Briefly, animals were deeply anesthetized at 24 hours after SAH and intracardially perfused with 60 mL ice-cold PBS followed by 60 mL of 10% paraformaldehyde through the upper part of the body. The whole brain was collected and fixed in 10% paraformaldehyde for 24 hours followed by 30% sucrose for three days. After being frozen at -80°C , the brains were cut into 10 μ m thick coronal sections on a cryostat (LM3050S; Leica Microsystems, Bannockburn, III, Germany). Double fluorescence labeling was processed as previously described (Enkhjargal et al., 2014). Brain sections were incubated overnight at 4°C with primary antibody including anti-GFAP (1:500, ab53554), anti-NeuN (1:500, ab177487), anti-iba-1 (1:500, ab107159) (Abcam, Cambridge, MA, USA), and anti-GLP-1R (1:50, sc-390774, Santa Cruz Biotechnology Inc.). Corresponding secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA, USA) were

incubated with the sections at room temperature for 2 hours. The sections were visualized and photographed with a fluorescence microscope (Leica Microsystems, Germany).

2.9. Fluoro-Jade C Staining

FJC staining was performed to detect degenerating neurons with a modified FJC ready-to-dilute staining kit (Biosensis, USA) according to the manufacturer's instructions (Shi et al., 2017; Xie et al., 2017). FJC-positive neurons were counted in six sections per brain at X200 magnification by an independent observer. Quantified analysis was performed with Image J software (Image J 1.4, NIH, USA). The data were presented by the average number of FJC-positive neurons in the fields as cells/mm².

2.10. Western blot analysis

Western blot tests were performed as previously reported (Sozen et al., 2009). Briefly, the whole left hemisphere was isolated and collected 24 hours after SAH (n=6 per group). Equal amounts of protein samples were loaded onto each lane of SDS-PAGE gel. After electrophoresis, the samples were transferred onto a nitrocellulose membrane, which was blocked with a blocking solution for 2 hours. The membrane was incubated at 4 °C overnight with the following primary antibody: anti-Bax (1:500, NBP1-28566, Novus Biologicals, CO, USA), anti-Bcl-2 (1:1000, ab59348), anti-PI3K (phospho Y607) (1:1000, ab182651) (Abcam, Cambridge, MA, USA), anti-Akt (1:1000, #9272), anti-phospho-Akt (Ser473) (1:1000, #9271), anti-Bcl-xl (1:1000, #2764) (Cell signaling, Boston, MA, USA), anti-GLP-1R (1:200, sc-390774), and anti-β-actin (1:5000, I-19) (Santa Cruz Biotechnology Inc., Texas, USA). Appropriate secondary antibodies (1:5000, Santa Cruz Biotechnology Inc., and anti-rabbit 2839792, EMD Millipore Corporation, MA, USA) were selected to incubate with the membrane for 2 hours in room temperature. The bands were detected with an ECL regents (Amersham Biosciences UK Ltd, PA, USA) and quantified by densitometry with Image J software (Image J 1.4, NIH, USA). β-actin was blotted on the same membrane as loading control.

2.11. Statistical analysis

Statistical analysis was performed with Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA). Data were presented as mean ± SD and analyzed by One-way analysis of variance (ANOVA) followed by multiple comparisons between groups using Tukey's post-hoc test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Mortality and SAH grade

Out of the 153 surgeries that were performed, 9 rats were excluded from the study due to mild SAH. The overall mortality of SAH was 14.88% (18/121, Fig. 2A). No rats died in Sham group. The SAH grading scores of all operated rats in Experiment 2 and Experiment 4 were assessed at 24 hours after SAH. There was no significant difference in SAH grading scores among the SAH groups (Fig. 2B).

3.2. Time course and spatial expression of GLP-1R after SAH

The endogenous expression of GLP-1R in left hemisphere was assessed by Western blots. There was a notable elevation of GLP-1R levels detected at 3 hours, which peaked at 24 hours and decreased at 72 hours after SAH when compared to Sham group ($P<0.05$, Fig. 3A). In that case, double immunohistochemistry of GLP-1R was performed on SAH rat at 24 hours after induction. Colocalization of GLP-1R with neurons (NeuN), astrocytes (GFAP), and microglia (Iba-1) was observed in ipsilateral basal cortex. GLP-1R was mainly expressed in neurons after SAH (Fig. 3B).

3.3. Ex-4 ameliorated short-term neurobehavioral deficiency after SAH

SAH induction significantly reduced neurological score on modified Garcia (left panel) and beam balance (right panel) at 24 hours after surgery in the SAH+Vehicle group compared with Sham group. The neurological impairments were remarkably recovered by administration of middle and high doses of Ex-4 treatment in the SAH+Ex-4 (3 μ g) and SAH+Ex-4 (9 μ g) groups, respectively, after SAH ($P<0.05$, Fig. 4A).

3.4. The effects of Ex-4 on physiologic parameters

The body weight loss in all groups were calculated with the body weight before surgery and body weight at 24 hours after surgery accordingly. High dose of Ex-4 significantly increased body weight loss in the SAH+Ex-4 (9 μ g) group compared to SAH+Vehicle group ($P<0.05$, Fig. 4B). Changes in blood glucose levels were monitored until 24 hours after surgery. SAH induction significantly increased blood glucose levels ($P<0.05$) before drug administration or at 1 hour after SAH. In spite of slightly lower levels of blood glucose found in SAH+Ex-4 (9 μ g) group compared to other SAH groups during the first hour after drug injection, there were no significant differences in blood glucose levels among the SAH groups ($P>0.05$). All Ex-4 treated groups had a trend of higher blood glucose levels compared to the Sham group at first 180 minutes after Ex-4 treatment, though no significant difference was detected ($P>0.05$) (Fig. 4C). The middle dose was chosen for further experiments since this dose of Ex-4 improved neurological score at 24 hours following SAH without decreasing body weight or blood glucose levels.

3.5. Ex-4 attenuated neuronal apoptosis after SAH

FJC was chosen to represent the severity of neuronal degeneration and apoptosis in the ipsilateral cortex at 24 hours after SAH (Fig. 5B). SAH induction significantly increased the number of FJC positive cells in SAH+Vehicle group when compared to Sham group ($P<0.05$) while administration of middle dose Ex-4 (3 μ g/rat) remarkably reduced this increase in the SAH+Ex-4 group ($P<0.05$) (Fig. 5A and C).

3.6. Ex-4 improved long-term neurobehavioral outcome after SAH

Animals in the SAH+Vehicle group had a significantly shorter falling latency in both 5 RPM and 10 RPM tests when compared to Sham group on day 7, 14 and 21 after SAH. Falling latency was also significantly improved in SAH+Ex-4 (3 μ g) group in both tests on the same time point ($P<0.05$, Fig. 6A).

In the water maze test, the travel distance and escape latency for animals to find the platform were significantly increased in the SAH+Vehicle group when compared to Sham group ($P<0.05$, Fig. 6B upper panels), however, a significantly shorter distance on Block 4 and a decrease in time to find the platform on days 3 & 4 were observed in the SAH+Ex-4 group compared to SAH+Vehicle group ($P<0.05$, Fig. 6B). In probe quadrant trail, SAH+Vehicle rats spent remarkably less time in the target quadrants when compared to Sham group, while the reference memory deficits were significantly improved with Ex-4 treatment in the SAH+Ex-4 group ($P<0.05$, Fig. 6B left lower panel). No significant difference of swimming velocity was detected among three groups ($P>0.05$, Fig. 6B right lower panel).

3.7. GLP-1R siRNA and LY294002 reversed the effects of Ex-4 on short-term neurobehavior at 24 hours after SAH

Intervention of GLP-1R siRNA and LY294002 did not result in significant difference in body weight loss in SAH+Ex-4+GLP-1R and SAH+Ex-4+LY294002 groups when compared to SAH+Ex-4+siRNA and SAH+Ex-4+DMSO groups, respectively, at 24 after SAH ($P>0.05$, Fig. 7A). However, administration of GLP-1R siRNA significantly decreased the neurological scores of Modified Garcia and beam balance in the SAH+Ex-4+GLP-1R siRNA group when compared to SAH+Ex-4+Scr siRNA group at 24 hours after SAH. Similarly, neurological scores in the SAH+Ex-4+LY294002 group were notably lower when compared to SAH+Ex-4+DMSO group at 24 hours following SAH ($P<0.05$, Fig. 7B, $n=6$ per group).

3.8. Ex-4 attenuated neuronal apoptosis through GLP-1R/PI3K/Akt pathway

After Ex-4 treatment ($3\mu\text{g}/\text{rat}$), expression of GLP-1R elevated dramatically when compared to both Sham (22.00 times) and SAH+Vehicle (5.76 times) groups ($P<0.05$, Fig. 7C and D left upper panel). Moreover, expression of PI3K, p-Akt, Bcl-xl, Bcl-2 was significantly decreased, while the expression of Bax increased remarkably in the SAH+Vehicle group when compared to Sham group at 24 hours after SAH. Consistently, expression of PI3K (2.26 times), p-Akt (7.40 times), Bcl-xl (1.75 times), Bcl-2 (1.70 times) was also significantly upregulated in the SAH+Ex-4 group when compared to SAH+Vehicle group at 24 hours after SAH (Fig. 7C and D). Expression of Bax, however, was remarkably downregulated after Ex-4 injection in the SAH+Ex-4 group ($P<0.05$, Fig. 7C and D right lower panel).

GLP-1R knockdown by i.c.v. injection of GLP-1R siRNA 48 hours before SAH induction significantly decreased expression of GLP-1R at 24 hours after SAH in the SAH+Ex-4+GLP-1R siRNA group when compared to SAH+Ex-4+Scr siRNA group ($P<0.05$, Fig. 7C and D left upper panel). Similarly, GLP-1R knockdown significantly decreased expression of PI3K, p-Akt, Bcl-xl, Bcl-2 and increased expression of Bax at 24 hours after SAH in SAH+Ex-4+GLP-1R siRNA rats ($P<0.05$, Fig. 7C and D).

Administration of LY294002, 30 minutes before induction of SAH, was used to assess PI3K involvement in the anti-apoptotic pathway of Ex-4 in the SAH model. The Western blot results showed that pretreatment with LY294002 significantly inhibited expression of PI3K in the SAH+Ex-4+LY294002 group when compared to SAH+Ex-4+DMSO group at 24

hours after SAH ($P<0.05$, Fig. 7C and D middle upper panel). Consistently, expression of the downstream molecule p-Akt was decreased, as well as Bcl-xl and Bcl-2 in the SAH +Ex-4+LY294002 group when compared to SAH+Ex-4+DMSO group at 24 hours after SAH. As a result, overproduction of Bax was observed at the same time in SAH +Ex-4+LY294002 rats ($P<0.05$, Fig. 7C and D, $n=6$ per group, $P<0.05$).

4. Discussion

In this present study, we assessed the potential neuroprotective effects of Ex-4 and explored the possible underlying mechanism of Ex-4 in EBI after SAH. We discovered that GLR-1R expression increased after SAH induction and GLP-1R can be expressed in neurons, astrocytes, and microglia cells following SAH. Administration of Ex-4 attenuated neuronal apoptosis and preserved neurological function after SAH. Additionally, we confirmed that PI3K/Akt signaling pathway is involved in the neuroprotective effects of Ex-4.

GLP-1R is an emerging potential target in CNS disease (Holscher, 2014; Muscogiuri et al., 2016; Salcedo et al., 2012). Previous studies revealed that GLP-1R was widely expressed in neurons and unless it involved inflammation, rarely in microglial cells (Alvarez et al., 1996; Gu et al., 2013). Our Western blot results indicate that expression of GLP-1R increased following SAH. In further double immunohistochemistry staining, expression of GLR-1R was observed in neurons, astrocytes, and microglia cells, especially in neurons of the ipsilateral cortex after SAH. Since inflammation is considered as one of the major pathological process in EBI following SAH (Chen et al., 2014), this finding was consistent with that of previous studies. It noteworthy that expression of GLP-1R increased to a higher level in the left hemisphere after delivery of Ex-4 when compared to vehicle group after induction of SAH. Effects of Ex-4 on GLP-1R expression complied with results of several previous studies that focused on other organ systems while utilizing the rat model (Fan et al., 2014; Yang et al., 2013; Zhang et al., 2011). As a result, the increased GLP-1R expression may magnify the potential therapeutic effects of GLP-1R agonists, such as Ex-4, in the SAH model.

GLP-1R agonists have been reported to be neuroprotective in various diseases of the CNS (Hou et al., 2012; Kuroki et al., 2016; Rachmany et al., 2013; Sato et al., 2013; Teramoto et al., 2011; Zhu et al., 2016). Since post-SAH cognitive dysfunction is considered to be just as devastating as its associated sensorimotor dysfunction, the present study performed both sensorimotor (Modified Garcia, beam balance, and rotarod) and cognitive (Morris water maze) evaluation in short-term and long-term to be more clinically relevant. The short-term outcome results reproduced the neuroprotective effects of Ex-4 that were previously induced in ischemic and traumatic brain injury models (Kuroki et al., 2016; Rachmany et al., 2013; Teramoto et al., 2011). Consistently, further investigation revealed that Ex-4 treatment has the potential to improve long-term sensorimotor dysfunction, spatial learning capacity, and even reference memory after induction of SAH.

Ex-4 was the first FDA approved GLP-1R agonist intended to treat adult type 2 diabetes mellitus (Briones and Bajaj, 2006). It is reported that GLP-1R agonists stimulate insulin secretion in a glucose-dependent manner and discontinue such stimulation when plasma

glucose levels fall below the normal fasting range (Parhofer, 2007; Rask et al., 2004). Being an anti-diabetic agent, Ex-4 associated hypoglycemia and weight loss have to be taken into consideration in future clinical applications. In order to further elucidate changes in blood glucose levels after SAH, to the best of our knowledge, our study is the first to record graphic evidence of blood glucose changes in an endovascular perforation model of SAH. According to the curve graph, acute hyperglycemia was found at 1 hour after SAH induction, possibly being a response to stress associated with surgery and SAH. Blood glucose levels of both sham-operated and SAH rats continued to decline in the first hour after SAH and reached the nadir at 24 hours post-SAH. There are various possible explanations that could account for the decline in blood glucose levels. One possibility is that surgery and SAH could increase the energy expenditure of the operated animals. Other possible explanations for the decline in blood glucose levels could be related to devascularization of the external carotid artery or that post-operative presence of a surgical wound might affect food intake capacity of the animals. Since blood glucose levels of all Ex-4 treated groups did not drop lower than the Sham group during the first 180 minutes after drug delivery and that Ex-4 had a half-life of approximately 2.4 hours (Egan et al., 2002), it is reasonable to believe that administration of Ex-4 did not increase the risk of hypoglycemia in endovascular perforation SAH model.

It is generally believed that neuronal apoptosis is a crucial process that is responsible for neurological impairment in EBI after SAH (Chen et al., 2014). The anti-apoptotic effects of Ex-4 have been reported in several non-SAH models (Teramoto et al., 2011; Urusova et al., 2004; Zhu et al., 2016). Two recent published studies suggest that the anti-apoptotic effects of GLP-1R agonists are mediated via PI3K/Akt signaling pathway (Zhang et al., 2016; Zhu et al., 2016). The present study echoed the results from the previous in that Ex-4 treatment attenuated apoptosis following SAH by as seen in results of FJC staining and Western blots. In Western blot results, Ex-4 treatment upregulated the expression of targeted protein on the upstream pathway (GLP-1R, PI3K, and p-Akt) as well as the anti-apoptotic molecular markers (Bcl-xl, Bcl-2) and downregulated the pro-apoptotic molecular marker (Bax). When GLP-1R was silenced by GLP-1R siRNA pretreatment, expression of GLP-1R, PI3K, p-Akt, Bcl-xl, Bcl-2 was suppressed and expression of Bax was enhanced, suggesting that anti-apoptotic effects of Ex-4 were abolished. Similarly, pretreatment with a PI3K inhibitor blocked the activation of PI3K and p-Akt, leading to decreased expression of anti-apoptotic markers (Bcl-xl, Bcl-2) and increased expression of pro-apoptotic marker (Bax). These findings suggest that activation of PI3K/Akt signaling pathway underlies the anti-apoptotic effects of Ex-4.

There were some limitations in our study. First, the present study did not rule out the possible neuroprotective effects of Ex-4 through preservation of the blood-brain barrier or alleviation of inflammation. Therefore, further studies must be conducted to explore the aforementioned possibilities. Second, it was previously reported that mitogen-activated protein kinase (MAPK) signaling pathway is thought to be involved in the anti-apoptotic effect of a GLP-1R agonist in an ischemic stroke model (Zhu et al., 2016). The results seen in GLP-1R siRNA and PI3K inhibitor pretreatment groups suggest that PI3K/Akt pathway is the most effective anti-apoptotic signaling pathway following induction of SAH. However, we were unable to identify the alternative role of MAPK in anti-apoptotic effects of Ex-4.

Third, we excluded the animals with mild SAH from our analyses, which might cause potential bias. However, previous study found induction of mild SAH (SAH grade 7) did not increase neurological deficits compared to sham animals (Sugawara et al., 2008). In that case, we believe animals with mild SAH could not represent the pathological progress in EBI after SAH which make it ineligible for further analyses. Another limitation in our study is related to the theory that increased insulin secretion is a potential neuroprotective factor. Although a previous study revealed that neuroprotective effects of Ex-4 were not mediated by insulin upregulation (Kuroki et al., 2016), this study did not measure insulin levels following induction of SAH. Lastly, the present study did not explore the mechanisms behind cognitive improvement as part of the long-term outcome.

In conclusion, this study revealed that administration of Ex-4 has the potential to improve both short-term and long-term neurological function in the SAH model. The neuroprotective role of Ex-4 in EBI after SAH is thought to be mediated by attenuation of neuronal apoptosis via GLP-1R/PI3K/Akt signaling pathway. If so, then Ex-4, the novel anti-diabetic drug, might have an additional utilization as an anti-apoptotic treatment in EBI following SAH, which may expand its application in cerebrovascular disease.

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Highlights

1. Ex-4 significantly improved neurological function after SAH.
2. GLP-1R mainly expressed in neurons after SAH.
3. Ex-4 did not increased risk of hypoglycemia after SAH in SD rats.
4. Ex-4 improved spatial learning capacity and reference memory after SAH.
5. The anti-apoptotic effect of Ex-4 was mediated via GLP-1R/PI3K/Akt pathway.

Fig. 1. Experimental design and animal groups

SAH, subarachnoid hemorrhage; WB, western blot; IHC, immunohistochemistry; h, hour; Ex-4, Exendin-4; DMSO, dimethyl sulfoxide; LY294002, PI3K specific inhibitor; Scr siRNA, scrambled siRNA.

Fig. 2. Mortality rate and subarachnoid hemorrhage (SAH) grade

(A) Mortality rates of all groups. (B) Representative image of SAH in the rat brain (upper panel) and summary of SAH grading scores (lower panel) of all groups in the short-term experiments (*Experiment 2* and *Experiment 4*). h, hour; Ex-4, Exendin-4; DMSO, dimethyl sulfoxide; LY294002, PI3K specific inhibitor; GLP-1R, glucagon-like peptide 1 receptor; siRNA, small interfering ribonucleic acid; Scr, scrambled.

Fig. 3. Endogenous expression of glucagon-like peptide 1 receptor (GLP-1R) after subarachnoid hemorrhage (SAH)

(A) Representative Western blots band of time course and densitometric quantification of GLP-1R after SAH. * $P < 0.05$ vs. Sham group. Data were presented as mean $\pm$ SD. n=6 per group. (B) Colocalization of GLP-1R with neurons (NeuN), astrocytes (GFAP) and microglia (Iba-1) at 24h after SAH. Nuclei are stained with DAPI (blue). Top panel indicates the location of staining in the brain (small black box). Scale bar=50 μ m, n=3 per group.

Fig. 4. The effects of Exendin-4 (Ex-4) on short-term neurobehavioral function and blood glucose level at 24h after subarachnoid hemorrhage (SAH)

(A) Ex-4 improved the modified Garcia score and beam balance Score at 24 hours after SAH. (B) The effects of Ex-4 on body weight loss at 24 hours after SAH. (C) The effects of Ex-4 on blood glucose change at 24 hours after SAH. Vehicle, sterile 0.9 % of NaCl
^{*} $P < 0.05$ and ^{**} $P < 0.01$ vs. SAH+Vehicle group; [#] $P < 0.05$ vs. Sham group; & $P < 0.05$ vs. Before SAH in indicated groups. $n = 6$ per group.

Fig. 5. The effects of Exendin-4 (Ex-4) on neuronal degenerating at 24 hours after subarachnoid hemorrhage (SAH)

(A) Representative microphotographs of Fluoro-Jade C staining (FJC)-positive neurons. (B) Quantitative analysis of FJC-positive cells was performed at the ipsilateral cortex. (C) FJC-positive neurons significantly increased after SAH induction. Vehicle, sterile 0.9 % of NaCl; Ex-4, 3 μ g of Exendin-4 treated 1 hour after SAH induction. * P < 0.05 vs. SAH+Vehicle group; # P < 0.05 vs. Sham group. Data were presented as mean $\pm$ SD. Scale bar = 50 μ m. n=3 per group.

Fig. 6. The effects of Exendin-4 (Ex-4) on the long-term neurobehavioral function after subarachnoid hemorrhage (SAH)

(A) Ex-4 increased falling latency in rotarod test on day 7, 14, and 21 after SAH. (B) Ex-4 improved water maze results on days 21–25 after SAH. Vehicle, sterile 0.9 % of NaCl; Ex-4, 3 μ g of Exendin-4 treated 1 hour after SAH induction. * P <0.05 vs. SAH+Vehicle group; # P <0.05 vs. Sham group. n =8 per group.

Fig. 7. GLP-1R siRNA and LY294002 pretreatment reversed the effects of Exendin-4 (Ex-4) after subarachnoid hemorrhage (SAH)

(A) The effects of GLP-1R siRNA and LY294002 on the body weight loss at 24 hours after SAH. (B) The effects of GLP-1R siRNA and LY294002 on neurological score at 24 hours after SAH in Ex-4 treated rats. (C) Representative Western blots images of downstream signaling molecules of GLP-1R. (D) Densitometric quantification of downstream signaling molecules of GLP-1R. Vehicle, sterile 0.9 % of NaCl; Ex-4, 3.0μg of Exendin-4 treated 1 hour after SAH induction; DMSO, dimethyl sulfoxide; Scr siRNA, scrambled siRNA.

* $P < 0.05$ between indicated groups; # $P < 0.05$ vs. Sham group. $n = 6$ per group.