Peptide hormone exendin-4 enhances neurogenesis in the dentate gyrus of photochemically induced cerebral ischemia model mice

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Abstract

Exendin-4 is a peptide resembling GLP-1 (glucagon-like-peptide 1) in the saliva of the glia monster, a poisonous lizard. Recently, exendin-4 has been reported that stimulates subventricular zone neurogenesis in the adult rodent brain. The author trying to evaluate the effects of exendin-4 on the neurogenesis in the hippocampus of photochemically induced cerebral ischemia model mice. Eight week-old male C57/BL6 mice were used. First, rose Bengal was injected via tail vein. After scalp incision, photothrombic lesion was made on right motor cortex with cool light radiation through optic fiber. Behavior test (adhesive-tape test) was performed to verify the appropriate brain injury. Daily injection of exendin-4 for 7 days was followed. Saline was injected in sham operation group. After 3 weeks of holiday, decapitation was performed. Exendin-4 treatment for one week significantly increases numbers of Ki-67 and BrdU immunoreactive cells in the hippocampus of ischemic model mice. The present study shows that exendin-4 is able to induce proliferation and differentiation in vivo of neural stem cells form the adult dentate gyrus and indicates a role for exendin-4 as modulator of neurogenesis of adult brain. **(J Med Life Sci 2014;11(2):89-94)**

Key Words : cerebral ischemia, exendin-4, neurogenesis

Introduction

New neuronal cells are continuously generated from neural progenitor/stem cells (NSC) in the adult mammalian brain and they are incorporated into the existing brain by a process known as adult neurogenesis[1]. This process occurs mainly in the subventricular zone (SVZ) and hippocampal dentate gyrus[1,2]. Pharmacological stimulation of endogenous NSC could lead to cell regeneration that may be beneficial in central nervous system (CNS) disorders[1-3].

The peptide hormone exendin-4, an agonist for the GLP-1 receptor (GLP-1R), was originated from the saliva of the lizard Heloderma suspectum[4,5]. Exendin-4 has been shown to promote neurotrophic or neuroprotective actions in the CNS[6]. In addition, it has been shown that exendin-4 can stimulate neurite outgrowth in PC12 cells; prevent cultured hippocampal neurons from apoptosis[6,7]. On the basis of the previous studies, the author examined whether exendin-4 was able to modulate neurogenesis after neuronal loss in the adult brain.

Watson et al. introduced photothrombotic infarcts in rats which are highly reproducible in size and location[8]. The procedure is that Rose Bengal, photosensitive dye, is administered intravenously and focal illumination of the skull is performed using a laser beam or cool light, which leads to local activation of the dye and free radicals. As a result, platelet aggregation and the coagulation cascades leads to occlusion of small vessels[9]. In mice, photothrombosis was achieved using an argon laser and intravenous Rose bengal injections[10]. The author used the irradiation with a conventional light source to induce the photothrombosis.

Materials and Methods

Adult (9 week-old), male C57/BL6 mice (Charles River Diagnostics, MA, USA) weighing 16-18 gm at time of arrival were used in all experiment. Mice were group housed in a temperature (20 ± 2℃) with access to food and water ad libitum (12-h light/dark cycle).

Animals were placed in a stereotactic frame. At the dorsal aspect of the head, the skull was exposed by a median incision of the skin. And bregma and lambda points were

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identified. A fiber optic bundle of a cold light source (Zeiss 1500 electronic, Jena, Germany) with a 3 mm aperture was centered using a micromanipulator at 0.4 mm posterior and 2 mm laterally from bregma. At this stereotactic position, the mouse sensorimotor cortex is located. The aperture of the cold light source was placed as close as possible to the skull to avoid scattering light that could cause variability. The brain was then illuminated through the intact skull for 5 min starting right after the injection of Rose Bengal (10 mg/kg) into tail vein. Afterwards, the skin was sutured and mice were allowed to awaken.

The injured area of cortex was verified by behavioral test. To perform behavioral test, a sleeve is created using a 3.0 cm piece of green paper tape (Fisher Scientific, Pittsburgh, PA, USA), 1.0 cm in width, and is wrapped around the forepaw so that the tape attaches to itself and that the fingers protrude slightly from the sleeve formed. If created correctly, the tape sleeve cannot be removed. The typical response is for the rat to vigorously attempt to remove the sleeve by either pulling at the tape with its mouth and/or brushing the tape with its contralateral paw.

The mice is then placed in its cage and observed for 30 seconds. The data collected represent the fraction of the 30-s observation period that the animal spends attending to the stimulus. The contralateral and ipsilateral limbs are tested separately. The test is repeated three times per testing day and the best two scores are averaged.

Two lesion groups consisting of 5 mice each were used in the study. One lesion group received saline, whereas the other lesion group received exendin-4 (0.1 μg/kg exendin-4 in PBS) daily for 7 days. All animals were also received BrdU (50 mg/kg) daily for 7 days. The animals were sacrificed 4 weeks after surgery.

Mouse were anaesthetized with pentobarbital sodium (50-60 mg/kg, i.p.) and perfused with 0.5 M phosphate buffered saline (PBS), and then fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were postfixed overnight at 4℃ in the same solution and soaked in 0.5 M PBS contains 30% sucrose for cryoprotection. Serial 30 μm-thick coronal sections were cut on a freezing microtome (Leica, Nussloch, Germany), and sorted in a cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB, pH 7.4) at -20℃ until ready for use.

For the immunohistochemical detection of BrdU, neuronal specific nuclear protein (NeuN), Ki-67, tissue sections of each mouse brain were taken from between -1.2 and -2.9 mm relative to bregma. For the immunolabeling of BrdU, the sections were processed as previously described (Moon et al. 2006). Free-floating sections were treated with 50% foramide, 280 mM NaCl, and 30 mM sodium citrate at 65℃ for 2 h, incubated in 2 M HCl at 37℃ for 30 min, and rinsed in 0.1 M boric acid (pH 8.5) at room temperature for 10 min. Sections were then incubated for 15 min in 1% H2O2 and then overnight at 25℃ in 0.3% Triton X-100 containing 0.5 mg/ml bovine serum albumin and one of the following primary antibodies: rat anti-BrdU antibody (1:1000 dilution; Roche, Mannheim, Germany), mouse monoclonal anti-Ki-67 antibody (1:1000; Sigma, St. Louis, MO, USA), or mouse monoclonal anti-NeuN antibody (1:1000; Chemicon, Temecula, CA, USA). The sections were then incubated for 120 min with anti-mouse or anti-goat secondary antibodies, as appropriate (1:200 each; Vector, Burlingame, CA, USA), followed by incubation with avidin-biotin-peroxidase complex (1:100; Vector) for 1 h at room temperature. Peroxidase activity was visualized by incubating the section with 0.02% 3,3-diaminobenxidine, and the sections were mounted on gelatin-coated slides. For immunofluorescent labeling, fluorescent anti-mouse Cy3 and anti-rat Cy2 secondary antibodies (1:500 dilution; Jackson ImmunoResearch, West Grove, PA, USA) were used, and the slices were observed under a confocal microscope (Axiovert LSM 510 META; Zeiss, Germany).

For all quantitative analyses, the investigator was blind to the experimental treatment. Raw cell counts and areas (as percentages) were analyzed using Student's t-test and one-way ANOVA followed by Scheffe's post hoc test. The data are presented as means ± S.M. Statistical significance was established at $p < 0.05$.

Results

After surgery, adhesive tape removal test was performed for evaluating the somatosensory infarct. The time of trying to detach the adhesive tape wrapping forelimb shows the activity of somatosensory cortex. The infarct was occurred in right somatosentory cortex of right hemisphere. The time of trying to detach the tape on left limb is significantly short than that of right limb (Fig. 1) which shows that the photothrombotic ischemia induce injury on the somatosentory cortex.

Ki-67 immunoreactivity shows the cell proliferation of the neural stem cells. BrdU immunoreactivity representing the incorporated BrdU injected 3 weeks ago shows the survival and maturation of neuron or glia. The immunoreactivity of Ki-67 and BrdU of exendin-4 treated mice increased in the dentate gyrus (Fig. 2). The present data indicates that

the treatment of exendin-4 for 1 week to photothrombotic ischemia model mice significantly increased the number of Ki-7 which shows the exendin-4 induce the increase of proliferation of neural stem cells in the dentate gyrus of ischemic brain and the treatment of exendin-4 for 1 week to photothrombotic ischemia model mice significantly increased the number of BrdU which shows the exendin-4 induce the increase of survival of neural stem cells and neurogenesis in the dentate gyrus of ischemic brain.

The number of BrdU immunoreactive cells in the dentate gyrus of exendin-4 treated mice was estimated. Exendin-4 treatment after photothrombotic ischemia significantly increased the BrdU immunoreactive cells in contralateral and ipsilateral dentate gyrus (Fig. 3)

The colocalization of BrdU incorporated cells with NeuN neural cell marker stain cells shows the differentiation of NSCs into the neuron in the dentate gyrus which is neurogenesis, the birth of new neruon. The double immunostaining shows that BrdU labeled NSCs (green) was colocalized with NeuN, neuronal marker, immunostaining (Fig. 4). Right magnified picture shows the white box area. The image of Z-section image confirms the colocalization of BrdU and NeuN immunostaining.

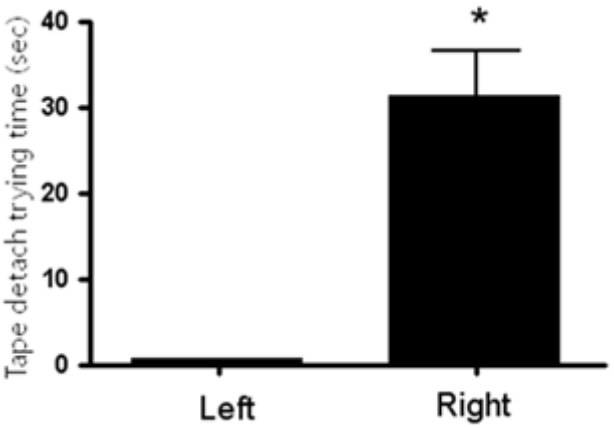

Figure 1. The adhesive tape removal test for evaluating the somatosensory infarct. The time of trying to detach the adhesive tape wrapping forelimb shows the activity of somatosensory cortex. The infarct was occurred in right somatosentory cortex of right hemisphere. The time of trying to detach the tape on left limb is significantly short than that of right limb. Values indicate the mean time of trying to detach the tape ± S.E.M. * indicates a statistically significant difference ($p < 0.05$) from the left limb based on Dunnet's C posthoc comparison.

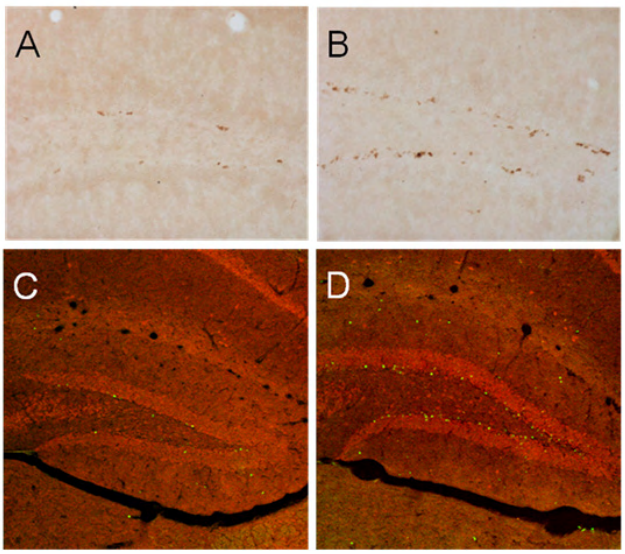

Figure 2. The change of reactivity of Ki-67 and BrdU in the dentate gyrus of exendin-4 treated mice. (A and B) The treatment of exendin-4 for 1 week to photothrombotic ischemia model mice significantly increased the number of Ki-7 which shows the exendin-4 induce the increase of proliferation of neural stem cells in the dentate gyrus of ischemic brain. (C and D) The treatment of exendin-4 for 1 week to photothrombotic ischemia model mice significantly increased the number of BrdU which shows the exendin-4 induce the increase of survival of neural stem cells and neurogenesis in the dentate gyrus of ischemic brain. A and C, ischemia + saline; B and D, ischemia + exendin-4.

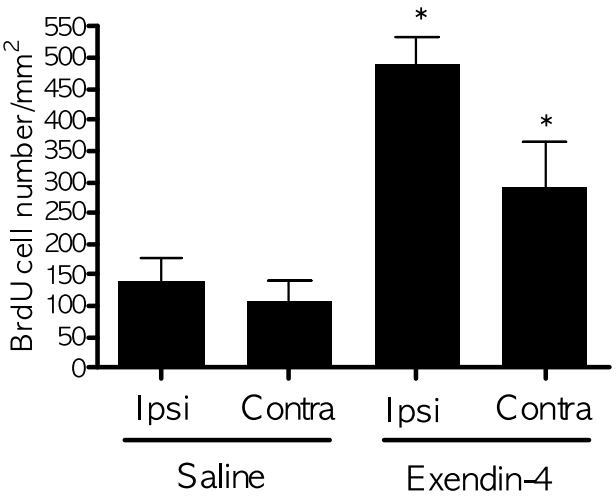

Figure 3. The change of number of BrdU immunoreactive cells in the dentate gyrus of exendin-4 treated mice. Exendin-4 treatment after photothrombotic ischemia increases the BrdU immunoreactive cells in contralateral and ipsilateral dentate gyrus. Values indicate the mean cell numbers/mm2±S.E.M.* indicate sastatistically significant difference ($p<0.05$) from the saline group based on Dunnet's C posthoc comparison.

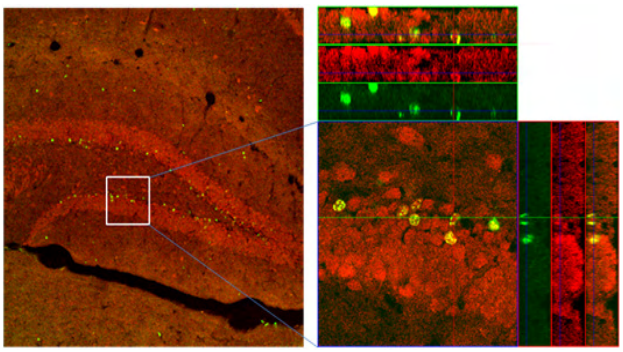

Figure 4. The differentiation of NSCs in the dentate gyrus. BrdU labeled NSCs (green) was colocalized with NeuN, neuronal marker, immustaining. Right magnified picture shows the white box area. The image was Z-section image from Zeiss confocal program.

Discussion

In this study, the author apply the new method for focal cortical ischemia which is photothrombotic infarctions using Rose Bengal. Photothrombotic infarctions in mice are highly reproducible in size and location. Therefore, they allow a reliable distinction between ischemic and nonischemic surrounding tissue. Usually, focal ischemia in mice has been achieved by local occlusion of the middle cerebral artery (MCA) at the level of the inferior cerebral vein. Infarcts induced by this method are usually rather large and complicated by a variable degree of edema formation. Therefore, the discrimination of nonischemic intact and infarcted tissue is difficult. Furthermore, the size of the cerebral infarct is highly dependent on the vascular anatomy of the MCA and collateral vessels which differs considerably between mouse strains[11]. CD-1 and Balb/c mice are considered relatively suitable strains for the direct MCA occlusion model, while reliable induction in C57BL/6 mice is difficult[11,12]. Cerebral photothrombosis in mice is minimally invasive and shares essential cellular responses with focal ischemia after middle cerebral artery occlusion[13]. It may provide a useful model to study the functional aspects of lesion-associated and remote molecular responses in transgenic mice lacking genes of interest in ischemia research.

In this study, the author used a modified version of the commonly used adhesive tape test as a measure of somatosensory dysfunction following experimental stroke[14]. The author found that in a cohort of animals with significant cerebral infarction, the modified version of adhesive tape test were able to adequately differentiate between sham-operated and MCA occluded animals at all time points.

Previous studies have shown that exendin-4 can promote beta-cell proliferation and islet neogenesis[15,16]. It has also been demonstrated that exendin-4 is able to enhance the differentiation of insulin-producing cells from embryonic stem cells[17]. Resently, the evidence for regenerative activity in the CNS or NSC stimulation has been reported[18], which demonstrated that an increase in overall cell number and in the number of cells undergoing mitosis was found in NSC cultures grown as neurospheres or as adherent monolayers and also in a rat embryonic striatal-derived stem cell line. The present study shows that exendin-4 also increase the neurogenesis in the dentate gyrus of the brain injured by

photothrombotic cortical ischemia.

To investigate the proliferation potential of NSC upon exendin-4 stimulation, the expression of certain markers for cell proliferation was studied. Exendin-4 treatment increased the number of Ki-67 immunoreactive cells in the dentate gyrus. This indicates that exendin-4 may modulate the cell proliferation NSC in dentate gyrus.

To study whether the neurogenic effects of exendin-4 on NSC also occur in neurogenic regions of the adult injured brain, photothrombotic ischemic adult mice were treated with exendin-4. The author found that exendin-4 promoted a significant increase in the number of cells that incorporate BrdU in the dentate gyrus as well as in the number of cells expressing the early neuronal marker NeuN in the dentate gyrus. However, it should be noted that our experiments do not rule out that survival of NSC could contribute to the neurogenic net effects by exendin-4.

Neurogenesis has been shown to occur throughout life in discrete regions of the CNS in mammals[19]. This slow physiological turnover of neurons in the adult brain suggests a functional role for NSC in the CNS. Endogenous neurogenesis can be regulated by, for example, physical exercise, enriched environment, injury (stroke), and growth factors[1,3]. For instance, after ischemic brain injury, it has been shown that VEGF, FGF-2 and GDNF increase neurogenesis from the adult dentategyrus. The new cells were able to integrate functionally[19,21,22].

Thus, the identification of compounds that can selectively trigger NSC to proliferate and to differentiate into desired phenotypes could be useful for the treatment of neurological diseases. Such compounds could be used for expanding subsets of defined cells populations for transplantation therapies or be used to stimulate adult neurogenesis.

Exendin-4 is used for the treatment of diabetes type II. Also, exendin-4 passes the blood-brain barrier, at least in mice, and could possibly have therapeutic CNS effects also after peripheral administration. It is interesting to note that exendin-4 has been shown to act as a neurotrophic factor, improving memory/cognition6[23]. Although additional experimental evidence is needed, this suggests a potential role for exendin-4 in the treatment of, e.g., stroke, Parkinson's disease, or Alzheimer's disease based on stimulation of neurogenesis. In conclusion, the author's results show that exendin-4 is able to promote adult neurogenesis in the dentate gyrus, and could be used for expanding subsets of defined cells populations that have therapeutic effects on brain injury.

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