**Exendin-4 antagonizes Aβ1-42-induced attenuation of spatial learning and memory ability**

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Received June 30, 2015; Accepted September 9, 2016

DOI: 10.3892/etm.2016.3742

**Abstract.** β-amylloid protein (Aβ) accumulation in cerebral centers involved in cognition and memory is a pivotal pathological feature of Alzheimer’s disease (AD). The onset process of type 2 diabetes mellitus (T2DM) has a number of similarities compared with AD. Thus, it is hypothesized that the pharmacological therapy employed for the treatment of T2DM may help to prevent and ameliorate the symptoms of AD. This study demonstrated that Exendin-4, which is a glucagon-like peptide-1 analogue which is used as a therapeutic drug for T2DM, markedly antagonized Aβ fragment-induced attenuation of spatial learning and memory ability, as indicated by a Morris water maze experiment. In addition, we investigated the potential underlying electrophysiological and molecular mechanisms. The results indicate that Exendin-4 rescued long-term potentiation from Aβ1-42-induced damage in the rat hippocampal CA1 region in vivo, and antagonized Aβ1-42-induced reduction of cyclic adenosine monophosphate and phosphorylated-cAMP response element-binding protein in rat hippocampal tissue using ELISA and western blot analysis, respectively. Thus, the results of the present study provide theoretical support for the application of Exendin-4 for improving AD.

**Introduction**

With the increase in the aging world population, the prevalence of major neurodegenerative diseases has become a serious public health problem worldwide, particularly Alzheimer’s disease (AD). AD is a degenerative, progressive and incurable neurological syndrome accompanied by a gradual decline in learning, memory and intelligence (1). A series of hypotheses have been proposed for the mechanism of AD, and it is now widely accepted that the deposit of amyloid β protein (Aβ) in cerebral centers involved in cognition and memory is the most important pathological feature of AD (2). There are two types of AD; familial and sporadic AD (3). Familial AD is associated with a genetic predisposition, which is associated by the production and deposition of Aβ (4).

Notably, the pathogenesis of another type of degenerative disease, type 2 diabetes mellitus (T2DM), has certain similarities with AD, such as Aβ deposits in the islets of T2DM patients. Further studies are necessary to assess whether therapeutic methods for T2DM could antagonize the neurotoxicity of Aβ peptides and ameliorate symptoms of AD patients. Glucagon-like peptide-1 (GLP-1) is a T2DM novel therapeutic drug has been suggested as a therapeutic target for AD (5). Due to the relatively short half-life of native GLP-1, which is easily degraded by dipeptidyl peptidase-4 (DPP-4), it has been limited in its clinical application (6). Exendin-4 is a GLP-1 analogue which is more slowly degraded, with a half-life of 9.57 h, and has been approved by the Federal Drug Administration as a therapeutic for T2DM (7).

Recently, studies have been published addressing the neuroprotective effect of GLP-1 analogues on neurodegenerative disorders (8,9). Our previous study indicated that Val8-GLP-1, another GLP-1 analog, was able to effectively antagonize synaptic dysfunction and intracellular Ca²⁺ overload induced by Aβ1-40 (10). This neuroprotection may be associated with the exhaustive regulation of synaptic transmission and intracellular calcium homeostasis by Val8-GLP-1 (11). Val8-GLP-1 prevented synaptic degeneration and reversed hippocampal synaptic plasticity in mice (12). In addition, Exendin-4 appears to increase the expression level of soluble polypeptide by enhancing the activity level of α-secretase of amyloid precursor protein (APP), thus antagonizing Aβ toxicity (13). The above findings suggest that GLP-1 analogs such as Exendin-4 may exert neuroprotective effects by improving synaptic function and antagonizing Aβ toxicity. However, whether Exendin-4 is able to mitigate spatial learning and memory damage resulting from Aβ fragment remains unclear. The present study investigated the effects of Exendin-4 on Aβ1-42-induced spatial learning and memory impairment, and on the overall behavior of rats in order to evaluate any neuroprotective effects induced by Exendin-4, and further investigated the underlying electrophysiological and molecular mechanisms.
Materials and methods

Experimental animal and reagents. A total of 80 adult male Sprague-Dawley rats (220-260 g) were used for the present study. All animals were provided by the Research Animal Center of Shanxi Medical University (Taiyuan, China). The study protocol received the approval of the Shanxi Animal Research Ethics Committee.

Aβ1-42 [Bachem (UK) Ltd, Saint Helens, UK] was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) then diluted with normal saline to 6.25x10⁻⁴ mol/l and incubated at 37°C for 36 h to form oligomers (14). Exendin-4 (Sigma-Aldrich) was diluted with normal saline to 2x10⁻⁴ mol/l. The administration was performed as follows: Intra-hippocampal injection 0.625 nmol (1 µl) Aβ1-42 (Aβ1-42 group); 0.2 nmol (1 µl) Exendin-4 (Exendin-4 group); Exendin-4 + Aβ1-42 group received Exendin-4 injection at 15 min following Aβ1-42 oligomers; and the control group received 1 µl normal saline injection. The intra-hippocampal injection was performed via a microinjection pump (KD Scientific, Inc., Holliston, MA, USA), with an injection rate of 0.1 µl/min. The experiments were then performed 5 days after.

Morris water maze test. The spatial learning and memory test was performed using a Morris water maze (MWM), as described previously (10). The MWM consists of a circular pool with a diameter of 150 cm (Zhenghua Biological Instrument Equipment Co., Ltd., Anhui, China), containing tap water at ~25°C. A underwater platform (14 cm in diameter) was placed under ~1.0 cm below the horizontal plane. At two weeks after drug injection, the hidden platform test was conducted. Animals underwent consecutive six-day trial periods, involving four trials per day using a random set of start locations. The spatial bias of the four quadrants was measured in the probe trial on the seventh day (10). Then the visible platform test was performed in order to measure the time of swimming to the platform (10).

Hippocampal CA1 region long-term potentiation (LTP) in vivo recording. Following the MWM test, the electrophysiological experiment was conducted as described in our previous study (10). In the present study, LTP was evaluated in vivo. The field excitatory postsynaptic potential (fEPSP) of the hippocampal CA1 region was recorded. Baseline fEPSP with test stimuli and LTP with high frequency stimulation (HFS) for at least 60 min were recorded. The averaged value of baseline fEPSP amplitude was taken as 100%.

Bicinchoninic acid (BCA) protein assay. Following the LTP experiment, the rats were sacrificed using an overdose of 25% urethane (5 ml/kg; Sigma-Aldrich) and the hippocampal tissues were dissected and frozen at -80°C. Then the tissues were homogenized with protease lysate, followed by centrifugation at 16,000 g for 10 min at 4°C, after which the supernatant was collected. The total protein was quantified using a BCA kit (Westang Biotech Co., Ltd., Shanghai, China), according to the manufacturer’s instructions. The BCA assay was conducted according to the manufacturer’s instructions. Absorbance was recorded at 562 nm using a microplate reader (MK3; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A standard curve was drawn and the total protein concentration was calculated.

Enzyme-linked immunosorbent assay (ELISA). Subsequently, an ELISA experiment was performed using a commercial kit (cat. no. F15181; Westang Biotech Co., Ltd.) to detect the expression of cyclic adenosine monophosphate (cAMP). The polystyrene ELISA plate was arranged and each group was marked clearly. Next, 100-µl standards of fold dilution were added to 8 wells of ELISA plate successively, then 100-µl samples were added to each well, with mixing and incubating at 37°C for 40 min, then washed 4-6 times and dried. Subsequently 50 µl distilled water and rabbit anti-rat cAMP antibody was added to each well (except the eighth standard well), mixed, incubated at 37°C for 20 min and washed with PBST. Next, 100 µl horse radish peroxidase working solution was added, mixed, incubated at 37°C for 10 min, washed and printed. Next, 100 µl substrate working solution was added at 37°C for 15 min, then subsequently 100 µl stop solution was added. Absorbance was recorded at 450 nm (within 30 min) using a microplate reader (MK3; Thermo Fisher Scientific, Inc.) and used to draw the standard curve. Finally, the content of cAMP was calculated. Absorbance was recorded at 450 nm (within 30 min) using a microplate reader and the content of cAMP was calculated according to the cAMP standard curve that was created with SkanIt Software (Thermo Fisher Scientific, Inc.).

Western blot analysis. The experiment was used to evaluate phosphorylated-cAMP response element binding protein (p-CREB) expression in rat hippocampal tissue. The bilateral hippocampus tissue (100 mg) was lysed in RIPA buffer (Beyotime Institute of Biotechnology, Nanjing, China). Loading sample (40 µg) was selected for separation using 12% SDS-PAGE, then transferred to a PVDF membrane (Whatman; GE Healthcare, Chalfont, UK). The membrane was blocked with 5% non-fat milk powder for 2 h and washed 3 times with 1× TBST buffer for 10 min. Then monoclonal primary antibodies against p-CREB (1:1,000; cat. no. 9198; Cell Signaling Technology, Inc., Danvers, MA, USA) were incubated with the membrane at 4°C overnight. Then the membrane was incubated with the corresponding secondary antibody for 1 h (peroxidase-conjugated goat anti-rat IgG; 1:3,000; cat. no. ZB-2307; Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). The blot was developed using a super enhanced chemiluminescence detection kit (cat. no. P1030; Applygen Technologies, Inc., Beijing, China), then the membrane was placed in a Kodak Image Station 400 (Kodak Company, Rochester, NY, Japan) for exposure and images of membrane signal bands were obtained. Image J software (image.nih.gov/ij/) was used to analyze the western blot bands.

Statistical analysis. Experimental data are presented as the mean ± standard deviation. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. One-way analysis of variance and repeated measures analysis of variance were used. Data were considered statistically significant when P<0.05.
Results

Intra-hippocampal injection of Aβ1-42 induced abnormal behavior. The experiment was performed using an MWM test to characterize the changes, if any, in rat spatial learning and memory after intra-hippocampal injection of Aβ1-42. Fig. 1A shows typical swim trajectories of rats in searching for the hidden platform in all groups in the sixth training day. The latencies (in sec, B and C) and distances (in centimeters, D and E) to find the hidden platform over six consecutive training days were shown for the control (n=15), Aβ1-42 (n=10), Exendin-4 + Aβ1-42 (n=10) and Exendin-4 (n=13) groups. Aβ1-42 increased the escape latencies and distances on training days 2, 3, 4, 5 and 6, while pretreatment with Exendin-4 significantly decreased the escape latencies and distances compared with Aβ1-42 group. Each point represents the mean ± standard deviation. **P<0.01 vs. control group. ***P<0.01 vs. Aβ1-42 group.

Exendin-4 mitigated the behavior ability caused by Aβ1-42. The subsequent results showed that there was no significant change of the typical swim trajectories between Exendin-4 and control group in the hidden platform test (Fig. 1A). Firstly, the application of Exendin-4 alone did not change the mean escape latencies and distances on any learning training day (Fig. 1B-E), the time percentage in the target quadrant remained consistent with control group in probe trial (Fig. 2A-C).

Furthermore, the neuroprotective impact of Exendin-4 on the behavioral ability was investigated. Mean escape latency and distance were decreased after pretreatment with Exendin-4 compared to the Aβ1-42 group in the learning ability test (Fig. 1). In the probe trial, the time in the target quadrant obviously prolonged compared with the Aβ1-42 group (Fig. 2C).

The latency and swimming speed were consistent among all groups during successive six days in the hidden platform.
and visible platform tests (Fig. 3). These data confirmed that the visual and motion ability of all rats was consistent, and the aforementioned behavioral experimental results indicate the effect of Exendin-4 and Aβ1-42.

**LTP was evidently reduced in the Aβ1-42 group.** The LTP experiment was conducted following the MWM test. Aβ1-42 significantly suppressed LTP in the rat hippocampus in vivo compared with control group, the average fEPSP amplitudes at 30, 60 and 90 min after HFS showed a significant difference compared to the control group (Fig. 4A and B).

The baseline fEPSP of intra-hippocampal injection of Aβ1-42 was observed. The result indicated that fEPSP without HFS was not significantly different during at least 90 min of recording in the Aβ1-42 group (Fig. 5). The results suggested that Aβ1-42 induced hippocampal LTP damage.

**Exendin-4 prevented Aβ1-42-induced impairment of LTP.** Similarly, Exendin-4 alone did not impact the baseline fEPSP (Fig. 5). However, Exendin-4 effectively prevented Aβ1-42-induced deficits of LTP. As shown in Fig. 4A and B, Aβ1-42 induced LTP inhibition was significantly antagonized by Exendin-4. The average fEPSP amplitudes at 30, 60 and 90 min post-HFS were significantly higher compared with the Aβ1-42 group.

**Exendin-4 antagonized Aβ1-42 induced decrease of cAMP and p-CREB in rat hippocampus tissue.** In order to investigate the molecular mechanism of antagonism of Exendin-4 for Aβ1-42, the levels of cAMP and p-CREB in the rat hippocampus were determined. We found that Aβ1-42 induced the cAMP level was significantly reduced compared with the control group, pretreatment of Exendin-4 antagonized the decrease of cAMP induced by Aβ1-42; however, the level of cAMP was not affected by Exendin-4 application alone (Fig. 6).

Subsequently, p-CREB expression in rat hippocampus tissue was determined in all groups. As shown in Fig. 7, Aβ1-42 evidently reduced the protein expression level of p-CREB.
p-CREB protein expression markedly improved after pretreatment with Exendin-4 compared with the application of Aβ1-42 alone. These results suggested that pretreatment with Exendin-4 may reverse the decreased p-CREB protein level induced by Aβ1-42. Furthermore, the protein expression level of p-CREB was not affected by Exendin-4 application alone. Thus, the present results imply that Exendin-4 may antagonize Aβ1-42 toxicity by mediating the cAMP/PKA/CREB pathway.

Discussion

In the present study, intra-hippocampal injection of Aβ1-42 was adopted to generate rat models, then behavioral, electrophysiological and molecular experiments were performed. The results imply that the impairment of learning and memory induced by Aβ1-42 was significantly reversed by Exendin-4 treatment. Furthermore, the electrophysiology and molecular mechanisms underlying the neuroprotective effect of Exendin-4 were investigated.

AD is a degenerative, progressive and incurable neurological disorder which is characterized by the gradual deterioration of cognitive function, neuropsychiatric symptoms and behavioral disturbances, including nutritional disorders and circadian rhythm disruption (15,16). There are three major pathological features associated with AD; neuronal loss, senile plaques formed by the deposition of amyloid β protein and neurofibrillary tangles (17,18). However, the exact pathogenesis of AD remains unclear, and thus a series of hypotheses have been proposed to account for the pathogenesis of AD, such as the Aβ hypothesis, cholinergic hypothesis, tau protein hypothesis and oxidative stress hypothesis, among which the Aβ hypothesis plays the most important role (19,20). The Aβ hypothesis suggests that the pathogenesis of various biological malfunctioning is associated with Aβ deposition, for example, Aβ oligomers extracted from human AD brain can inhibit LTP, reduce dendritic spine density and disrupt memory and learning in vivo when directly injected into a mouse hippocampus (21). Aβ is derived from the precursor protein APP,
which generates various polypeptides via sequential cleaving by α-secretase and β-secretase (22). Aβ is an insoluble polypeptide which is generated by the β-secretase pathway, and can induce oxidative stress and Ca\(^{2+}\) uptake. Oxidative stress can in turn active apoptosis and initiate glial cells to produce large quantities of inflammatory mediators and other toxic substances, resulting in irreversible neuronal damage (23). Moreover, Ca\(^{2+}\) may be releases from presynaptic neurotransmitters and influx into the postsynaptic membrane, which is closely associated with learning and memory (24).

By contrast, Aβ peptide includes numerous fragments, including Aβ1-40, Aβ1-42, Aβ31-35 and Aβ25-35 (25,26). Among these, Aβ1-42 exhibits the strongest toxicity owing to its hydrophobicity and aggregation (27). Therefore, Aβ1-42 was adopted for the present experiments. Formerly, intra-cerebroventricular injection was widely used to investigated the toxicity of Aβ1-42 in numerous studies (28,29); however, this delivery method has numerous disadvantages, including drug diffusion, inaccurate positioning and relatively high cost (10,30). Intra-hippocampal administration effectively avoided these shortcomings and shortened delivery time significantly in the present study.

Following intra-hippocampal injection, rats underwent an MWM experiment. Over the course of six consecutive days of spatial learning ability testing, the escape latencies and distances of Aβ1-42 group increased significantly compared with the control group. Approximately equal time was spent in the target quadrant compared with any other quadrant in the Aβ1-42 group in the probe trial. It is thus indicated that the spatial learning and memory impairment were induced by Aβ1-42. Consistent with this, inhibitory avoidance analysis (a fear conditioning-based task) in a previous study suggested that Aβ1-42 caused the damage to learning and memory ability (31). Therefore, protection against neurotoxicity of Aβ is hypothesized to be a therapeutic target for the treatment of AD patients. Several studies have indicated approaches to decreasing Aβ neurotoxicity, such as reducing Aβ generation, increasing Aβ degradation and restraining Aβ toxicity (32,33); however, there is at present no efficacious drug directed against Aβ neurotoxicity. The purpose of the present study was to investigate whether Exendin-4 exhibits neuroprotective effects against Aβ peptide neurotoxicity.

A previous study suggested an association between AD and T2DM, which is another chronic degenerative disease (34). An investigation revealed that 85% of AD patients were diagnosed with T2DM or accompanied by elevated fasting blood glucose (35). Furthermore, the risk of patients with T2DM suffering from AD is twice that of the normal population (36). Notably, Aβ deposition, the typical pathological change in AD patients, is similar to the characteristic accumulation of Aβ in the islet cells of patients with T2DM (37). Therefore, researchers have suggested that AD associated with high blood glucose be considered 'type 3 diabetes mellitus' (38). Thus, pharmacological therapy intended for the treatment of T2DM may additionally help to prevent and improve the symptoms of AD. Insulin, the primary therapy for T2DM, is not applicable as an AD treatment, as insulin-degrading enzyme can recognize and combine the same domain which exists in insulin and Aβ (39). Moreover, insulin injection can reduce the combination between Aβ and IDE, facilitate Aβ deposition in brain, and ultimately exacerbate the neurotoxicity and induce clinical symptoms (40). Furthermore, AD patients with normal blood glucose may sustain glucopenia due to the fact that insulin can lower euglycemia (41). However, GLP-1 and its analogs may promote insulin synthesis and secretion, and do not affect normal blood glucose (42). The polypeptide chain of GLP-1 containing 30 amino acids can be decomposed by DPP-4, therefore the plasma half-life of native GLP-1 is <2 min (6,43). GLP-1 analogs are similar to native GLP-1 as they exhibit similar chemical structure and biological activity (44). Fortunately, certain GLP-1 analogs are not easily biodegraded by DPP-4, which is attributed to the fatty acid side chains, so its plasma
half-life is longer than that of native GLP-1 and may cross the blood-brain barrier (45). Exendin-4, derived from American Gila monster’s (Heloderma suspectum) saliva, is the main GLP-1 analogue approved for clinical application (46). Approximately 53% homology exists between Exendin-4 and mammalian GLP-1, ad Exendin-4 has high affinity to GLP-1 receptor, whose plasma half-life is 9.57 h (47). Studies have found that Exendin-4 protected the primary human SH-SYSY cells and rat hippocampal neurons against glutamate excitotoxicity (48), as well as abating the cognitive impairment induced by traumatic brain injury (48). However, whether Exendin-4 can mitigate the damage of spatial learning and memory derived associated with Aβ remains unknown. The results of the present study demonstrated that Exendin-4 was associated with a marked reduction in Aβ1-42-induced learning and memory damage in a rat model. In the behavioral experiment, pretreatment of Exendin-4 decreased mean escape latency and distance travelled by the rats, compared with the Aβ1-42 group in the learning test, and the time in the target quadrant obviously prolonged compared with the Aβ1-42 group in the memory trial. In order to investigate the mechanism underlying the neuroprotective effect of Exendin-4, we conducted further electrophysiological and molecular biological experiments.

As an important electrophysiological mechanism of learning and memory, LTP is a long-lasting increase in synaptic efficacy which follows high-frequency stimulation of afferent fibers (49). Our previous study showed that Aβ1-40-induced damage of late phase LTP, learning and memory were antagonized by Valβ-GLP-1 (10). In the present study, we performed electrophysiological experiments and proved that Aβ1-42-induced a reduction of LTP could be reversed by Exendin-4. Baseline fEPSP in the CA1 area of hippocampus was unchanged by Aβ1-42 or Exendin-4 alone, which indicates that Exendin-4 regulates learning and memory impairment of AD model mediated by modifying impaired LTP.

Furthermore, synaptic plasticity, neurogenesis, learning and memory are closely associated with cAMP/PKA/CREB signaling pathway in the central nervous system (50,51). CREB is an important nucleoprotein belonging to activating transcription factor and CREB proteins are activated by phosphorylation at Ser133 (p-CREB) from various kinases that are activated by the increased intracellular cAMP (52). A previous study showed that cAMP/PKA/CREB signaling pathway had a significant effect on memory formation, particularly hippocampal-dependent LTP (53). Increased cAMP, activated MAPK and p-CREB during rapid eye movement sleep ultimately contributed to memory consolidation (54). In addition, a previous study has confirmed that Aβ25-35-treated rats displayed decreased levels of p-CREB (55). The results of the present study indicate that Exendin-4 could reverse Aβ1-42-induced decline of cAMP and p-CREB, which suggested that Exendin-4 ameliorates Aβ1-42-induced damage to learning and memory, potentially by modifying the cAMP/PKA/CREB signaling pathway.

In conclusion, the study demonstrated that Exendin-4 may exert a neuroprotective effect in the experimental rats, and further revealed possible underlying electrophysiological and molecular mechanisms. These findings provide theoretical support for further investigation into the use of Exendin-4 as a drug for the prophylaxis and treatment of AD.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81471343) and Science and Technology Innovation Fund of Shaxi Medical University (grant no. 01201307).

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