Peptidic exenatide and herbal catalpol mediate neuroprotection via the hippocampal GLP-1 receptor/β-endorphin pathway

Yu Jia, Nian Gong, Teng-Fei Li, Bin Zhu, Yong-Xiang Wang*

King's Lab, Shanghai Jiao Tong University School of Pharmacy, Shanghai, China

**Abstract**

Both peptidic agonist exenatide and herbal agonist catalpol of the glucagon-like peptide-1 receptor (GLP-1R) are neuroprotective. We have previously shown that activation of spinal GLP-1Rs expresses β-endorphin in microglia to produce antinoception. The aim of this study was to explore whether exenatide and catalpol exert neuroprotection via activation of the hippocampal GLP-1R/β-endorphin pathway. The rat middle cerebral artery occlusion model was employed, and the GLP-1R immunofluorescence staining and β-endorphin measurement were assayed in the hippocampus and primary cultures of microglia, neurons and astrocytes. The immunoreactivity of GLP-1Rs on microglia in the hippocampus was upregulated after ischemia reperfusion. Intracerebroventricular (i.c.v.) injection of exenatide and catalpol produced neuroprotection in the rat transient ischemia/reperfusion model, reflected by a marked reduction in brain infarction size and a mild recovery in neurobehavioural deficits. In addition, i.c.v. injection of exenatide and catalpol significantly stimulated β-endorphin expression in the hippocampus and cultured primary microglia (but not primary neurons or astrocytes). Furthermore, exenatide and catalpol neuroprotection was completely blocked by i.c.v. injection of the GLP-1R orthosteric antagonist exendin (9–39), specific β-endorphin antiserum, and selective opioid receptor antagonist naloxone. Our results indicate, for the first time, that the neuroprotective effects of catalpol and exenatide are GLP-1R-specific, and that these effects are mediated by β-endorphin expression probably in hippocampal microglia. We postulate that in contrast to the peripheral tissue, where the activation of GLP-1Rs in pancreas islet β-cells causes secretion of insulin to perform glucoregulation, it leads to β-endorphin expression in microglial cells to produce neuroprotection and analgesia in the central nervous system.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Glucagon-like peptide-1 receptors (GLP-1Rs) are distributed in various tissues, including the pancreatic islets and lungs, the cardiovascular system, and the central nervous system, but not in the skeletal muscle [1–6]. In the central nervous system, GLP-1Rs are located in the cortex, neocortex, hypothalamus, brainstem, hippocampus, cerebellum, and spinal cord [2,7–12]. GLP-1Rs modulate central neuronal activity and protect against neuronal damage induced by various insults [13–15]. Rapidly accumulating evidence suggests that the GLP-1R agonists exenatide and GLP-1, used for the treatment of type 2 diabetes mellitus, also display neuroprotective properties in multiple models of neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, amyothrophic lateral sclerosis, peripheral neuropathy, multiple sclerosis, ischemia, and stroke [11,14,16–22]. However, the mechanisms and signal transduction underlying the neuroprotective effects of GLP-1R are not clear. It is reported the neuroprotective effects of GLP-1R stimulation on cultured primary cortical neurons, suggesting a neuronal mechanism [14]. In contrast, exenatide was reported to exert neuroprotection via blocking 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced microglial activation and reducing the expression of matrix metalloproteinase-3 and stimulated proinflammatory cytokines (TNF-α and IL-1β) in substantia nigra pars compacta and striatum [21]. Activation of GLP-1Rs was also reported to increase the expression of the proinflammatory cytokines in the hypothalamus and the hindbrain [23], or have no effect on proinflammatory cytokine expression in the spinal cord or cultured primary microglia [24]. On the other hand, exenatide was shown to stimulate neurogenesis, which serves a
possible mechanism by which the peptide might restore function in a damaged system [16,18].

We recently identified the GLP-1R/β-endorphin antinociceptive pathway in the spinal cord. GLP-1Rs were exclusively expressed in the spinal dorsal horn microglial cells, and were consequently upgraded accompanying microglial activation following peripheral nerve injury. The peptidic agonists GLP-1 (7–36) and exenatide, non-peptidic agonist WB4-24, and herbal agonist shanzhiside methylster of GLP-1 produced anti-hypersensitivity in a variety of animal models of chronic pain, including neuropathic pain, inflammatory pain, bone cancer pain, and painful diabetic neuropathy. These GLP-1R agonists stimulated β-endorphin expression in spinal dorsal horn or cultured primary microglial cells from the spinal cord. The antinociceptive effects of exenatide, GLP-1, WB4-24 and shanzhiside methylster were completely blocked by the microglial inhibitor minocycline, specific β-endorphin antisemur (but not the dynorphin A antisemur), and opioid receptor antagonist naloxone or selective μ-opioid receptor antagonist CTAP [2,24,25]. It is not known whether the GLP-1R/β-endorphin pathway also exists in the hippocampus, and whether exenatide produces neuroprotection via this pathway.

Catalpol is an important iridoid glycoside purified from Rehmannia glutinosa Libosch, which has been widely used as a traditional Chinese herbal medicine for the treatment of aging diseases and stroke. In the previous studies, catalpol significantly attenuated apoptosis, rescued hippocampal CA1 neurons, and reduced cognitive impairment in the transient focal and global cerebral ischemic models in gerbils [26,27]. It was also recently reported that catalpol significantly facilitated neurological function recovery, reduced infarction volume, and increased cerebral blood flow in a mouse stroke model [28]. It improved memory and protected the forebrain neurons from neurodegeneration in a neurodegenerative mouse model [29]. These results indicated that catalpol was an active ingredient which could reduce ischemic damage and enhance memory. We recently discovered that catalpol was an orthosteric agonist of GLP-1Rs that functioned similarly in humans and rats and presumably acted at the same binding site as exendin (9–39). In addition, intrathecal injection of catalpol blocked formalin-induced tonic pain and its antinociceptive effect was completely blocked by exendin (9–39) [30]. It is not known whether the neuroprotective effect of catalpol is via activation of GLP-1Rs in the brain and subsequent expression of β-endorphin.

In this study, we explored the possible involvement of the hippocampal microglial GLP-1R/β-endorphin pathway in the neuroprotective effects of both exenatide and catalpol. We first confirmed the neuroprotective effects of intracerebroventricular (i.c.v.) injection of exenatide and catalpol in a rat transient ischemia/reperfusion model. We then tested whether the GLP-1R antagonist exendin (9–39) blocked the i.c.v. injection of catalpol and exenatide-induced neuroprotection. We further examined whether treatment with exenatide and catalpol stimulated β-endorphin expression in the hippocampus and primary cultures of microglia. We finally tested whether i.c.v. injection of the specific β-endorphin antisemur and selective opioid receptor antagonist naloxone reversed the neuroprotective effects of exenatide and catalpol. Our results suggest, for the first time, that exenatide and catalpol exert their neuroprotective effects via stimulation of the GLP-1R/β-endorphin pathway probably in hippocampal microglia.

2. Materials and methods

2.1. Animals and drugs

Male adult and 1-day old neonatal Wistar rats (250–300 g), purchased from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China), were maintained under temperature- and light-controlled conditions (20–24 °C, 12-h light/dark cycle) with continuous access to food and water. The rats were acclimated to the laboratory environment for 3–5 days before the study began. All of the experiments were performed in accordance with the Animal Care and Welfare Committee of the Shanghai Jiao Tong University, and followed the animal care guidelines of the National Institutes of Health.

Exenatide and exendin (9–39) were obtained from Kajjie Bio-Pharmaceutical Co. (Chengdu, China) and Shanghai TASH Biotechnology Co. (Shanghai, China). Catalpol (purity ≥98% by HPLC) and naloxone hydrochloride were purchased from Biopurity Phytochemicals (Chengdu, China) and Sigma–Aldrich (Shanghai, China), respectively. The rabbit polyclonal antibodies neutralizing β-endorphin was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). Based on the manufacturer’s information, the β-endorphin antisemur was specific to β-endorphin and did not cross-react with methionine-enkephalin, leucine-enkaphalin, dynorphin A or B, γ-endorphin, ACTH or α-melanocyte-stimulating hormone. All of the drugs or reagents were freshly dissolved in saline before use.

2.1.1. Immunofluorescence staining [2]

Single and double immunofluorescence labeling of the GLP-1R and microglia were performed on the hippocampi. Rats were anesthetized by pentobarbital injection (30 mg/kg) and intracar-dially perfused with 500 mL normal saline followed by 300 mL of 4% paraformaldehyde (wt/vol) in phosphate buffered saline. The brain of each rat was rapidly removed and the complete hippocampus was collected and fixed in 4% buffered paraformaldehyde at 4 °C for 24 h. Tissues were entrapped in OCT embedding agent (Leica Microsystems, Wetzlar, Germany) and cut into 40 μm-thick frozen sections, which were then incubated with GLP-1R antibody (ab119287, 1:100, rabbit polyclonal, Abcam, Cambridge, UK) and OX42 antibody (ab1211, 1:200, mouse polyclonal, Abcam) for microglia at 4 °C for 24 h. The GLP-1R antibody was tested for optimal dilution and nonspecific staining [2]. The GLP-1R and OX42 antibodies were visualized with Alexa 555-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen, Grand Island, NY, USA) and Alexa 488-conjugated goat anti-mouse secondary antibody (1:200, Invitrogen), respectively. DAPI (4′,6-diamidino-2-phenylindole, Sigma–Aldrich) staining was used to determine the cell nuclei.

The OX42 and GLP-1R-immunopositive cell profiles were quantified using a Leica TCS SP5II confocal microscope (Leica Microsystems). Identical confocal acquisition parameters were set for GLP-1R and OX42 staining, including objective, laser power, photomultiplier gain and offset, emission window, pinhole, and speed. Images of the CA1 and dentate gyrus regions were captured under ×10 magnification. All positively stained cells in the stratum lacunosum moleculare layer of the CA1 region were measured using a computer-assisted image analysis program (Image J Software, National Institutes of Health, MD, USA) in a blinded fashion. Low and high thresholds were set to exclude background fluorescence and to include immunofluorescent intensity measurements only from positively stained cell surfaces. We determined the thresholds visually by moving the cursor in the displayed histogram until all the background pixels in the image were darkened; the intensity value at the position of the cursor was taken as the threshold value for the channel. The cell showing a two-fold more intense staining than the average background was considered positive. A colocalization analysis was performed using Image J software with a Colocalization Finder to generate images in which colocalized pixels appeared as white. The same threshold value configuration was used to measure all of the surface areas in each experimental group at the same time. The measured areas were
imported into the Excel automatically and the Image J program was calibrated to provide standardized area measurements. The GLP−1R and OX42−immunolabeled surface areas were measured in brain slices (thickness: 40 μm, −3.6 mm to −4.3 mm to the bregma) by a researcher blinded to the experimental conditions. The averaged percentage immunolabeled surface area was the fraction of the positive immunofluorescent surface area of the hippocampus CA1 area from three sections of each brain. The data from six rats from each group were then calculated.

2.1.2. Primary cultures of neurons and glial cells [2]

Neurons and glial cells were isolated from the hippocampus of 1-day-old neonatal rats. The isolated hippocampi was minced and incubated with trypsin. Dissociated cells were suspended in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum and penicillin (100 U/mL) and streptomycin (100 μg/mL). For the neuronal culture, cell suspensions were plated in plates pre-coated with poly-l-lysine (100 μg/mL). After 1.5 h of incubation, the medium was changed to Neurobasal (Invitrogen) containing B27 supplement and 0.5 mM glutamine for further culture. All experiments were initiated 5–6 days after plating. Harvested neurons showed a purity >85%, as determined by NeuN immunoreactivity.

For glial cell cultures, cell suspensions of minced hippocampi were plated in 75-cm² tissue culture flasks (1 × 10⁷ cells/flask) pre-coated with poly-l-lysine and maintained in a 5% carbon dioxide incubator at 37 °C. After culture for 8 days, microglial cells were prepared as floating cell suspensions by shaking the flasks at 250 rpm for 2 h. The aliquots were transferred to plates and unattached cells were removed by washing with serum-free DMEM. Harvested microglial cells showed a purity >95%, as determined by OX42 immunoreactivity. After culture for 11 days, astrocytes were prepared as floating cell suspensions by shaking the flasks for 2 h followed by incubation with 10 mL of 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen) in a cell incubator for 15 min to separate the oligodendrocytes from the astrocytes. After trypsin neutralization with 10 mL of complete media, the floating cell suspensions were discarded. A nearly intact layer of astrocytes in the bed layer were then trypsinized and sub-cultured conventionally. Prepared astrocytes showed a purity >90%, as determined by GFAP immunoreactivity.

2.1.3. β-Endorphin measurement [2]

The β-endorphin concentrations were measured in the ipsilateral hippocampi from ischemic rats and primary cultures of neurons and glial cells. The ipsilateral hippocampi were taken, homogenized (4000 rpm) for 15 s in 10 mM Tris−HCl (pH 7.4) (1 g tissue/5 mL Tris−HCl) by a homogenizer (Fluko Equipment Co., Shanghai, China), and centrifuged (4000 rpm) at 4 °C for 15 min. The protein content was measured using a standard BCA protein assay (Beyotime Institute of Biotechnology, Jiangsu, China). For primary cultures, neurons, astrocytes, and microglia originated from the hippocampi of 1-day-old neonatal rats were placed in 24-well plates (5 × 10⁵ cells/well) and washed twice with 1 mL of warm DMEM containing 2 mg/mL of bovine serum albumin and 15 mM N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid. Cells were exposed to different concentrations of exenatide and catalpol for 2 h and supernatant samples were processed.

The β-endorphin concentration was determined using an enzyme-linked fluorescent immunosays kit (Phoenix Pharmaceuticals, CA, USA) with the linear standard curve of 29.6−608 pg/mL. The cross-activity of the assay included α-endorphin (100%) and γ-endorphin (60%), but not methionine-enkephalin (0%) or leucine-enkephalin (0%), according to the manufacturer’s information. The relative fluorescence units were measured with a fluorescence microplate reader (Thermo Labsystems, Vantaa, Finland) and the concentrations of samples were determined by comparison with a calibration curve running at the same time.

2.1.4. Rat I.C.V. catheterization and injection [31]

The rats were anaesthetized with pentobarbital sodium (50 mg/kg) by intraperitoneal injection and placed on a stereotaxic apparatus (Stoelting Company, Wood Dale, IL). After the skull was exposed, a 24-gauge guide cannula (0.55 mm) was stereotactically implanted 3 mm above the left lateral ventricle (anterior−posterior: −1.1 mm from the bregma; mediolateral: −1.5 mm; dorsal:ventral: +2.8 mm below the surface of the skull). The guide cannula was anchored by enhanced glass plastic cement type I, which is used in dentistry. The guide cannula was kept viable with the use of a dummy cannula to prevent it from blocking. The animals were allowed to return to their home cages and recover for at least 7 days before undergoing the middle cerebral artery occlusion (MCAO) operation. The control vehicle and test drugs in 5 μL were slowly injected using a 30-gauge cannula extended 1 mm beyond the guide cannula and connected to a 10-μL microsyringe via a polyethylene tube, which remained in place for 30 s to allow for diffusion.

2.1.5. The rat MCAO model [32]

MCAO, an animal stroke model that closely resembles the most common form of human stroke, was induced in the left side using the intraluminal filament technique [14]. Briefly, the male adult Wistar rats (250−300 g) were anaesthetized by inhalation of 1.0% isoflurane and their body temperature was maintained at 37−38 °C using a heating pad. The common and external carotid arteries were ligated, and the internal carotid artery was temporarily closed. Two slipknots were made at the external carotid arteries, near to the bifurcation site of the common carotid arteries, and a small hole was cut on the external carotid arteries between the two knots. A 4-0 nylon monofilament (Shadong Biotech, Beijing, China) coated with a silicone tip was advanced 1.8−1.9 cm from the bifurcation site, through the internal carotid artery, to the origin of the middle cerebral artery. The slipknot at the external carotid arteries was tightened and the wound was closed. The animals were allowed to wake up and were placed in their cage. After occlusion for 60 min, they were anaesthetized again and the filament was withdrawn for reperfusion. Two days later, the rats underwent neurobehavioral testing and evaluation of the infarct areas.

2.1.6. Neurobehavioral testing [33]

The neurological status of each rat was evaluated 2 days after ischemic reperfusion according to the Zuo-Longa neurological grading scale. Grades were based on the degree of contralateral hemiparesis: 0 = no observable deficit; 1 = failure to extend the ipsilateral forepaw; 2 = circling to the ipsilateral side; 3 = falling to the ipsilateral side; and 4 = unable to walk spontaneously and with a depressed level of consciousness.

2.1.7. Evaluation of infarct areas [34]

The animals were killed 48 h after the MCAO reperfusion and evaluation of the neurological assessment. The brains were removed rapidly, with the first knife point at the midpoint between the anterior pole of the brain and the optic chiasma. The brains were sliced into consecutive 6 sections of 2.0 mm-thick from anterior to posterior, and incubated in 2% w/v 2,3,5-triphenyltetrazolium chloride (TTC, Sigma–Aldrich) at 37 °C for 15 min. The stained slices were then fixed by immersion in phosphate-buffered 4% paraformaldehyde for 8–9 h. To determine the infarct area by TTC staining, the images of the 6 sections were shot using a Sony DSC-HX7 camera with the same parameter settings, and the acquired images were processed by a blinded observer using the Adobe Photoshop CS2 program (Adobe System, CA, USA). To correct the effects
of cerebral edema and differential shrinkage resulting from tissue processing, the infarction area in each section was calculated by subtracting the area of the healthy, uninfarced (TTC-stained) tissue in the ipsilateral hemisphere from the area of the contralateral hemisphere as described previously [35]. The infarction area of each brain slice was expressed as a percentage (%) of the contralateral hemisphere area, i.e., uninfarced area of ipsilateral hemisphere/contralateral hemispheric area × 100%. The infarction area of the brain was calculated by averaging the infarction areas of all 6 sections.

2.2. Data analysis and statistical evaluation

Data are presented as means ± SEM, and there were no missing data. The sample size of the animal studies was chosen on the experience that six to eight rats might be sufficient to show statistical significance for the more than 40% differences (from the preliminary experiments) of infarct areas, neurobehavioral deficits, GLP-1R upgrade, and β-endorphin increment compared to the respective controls, with reasonable standard deviations. Statistical significance was evaluated by paired Student t-tests or one-way analysis of variance (ANOVA), as indicated. A Student–Newman–Keuls post-hoc test was performed when a statistically significant drug effect was observed. The criterion P value for statistical significance was 0.05. All data calculations and statistics analysis were performed in the GraphPad Prism Program (version 5.01, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The immunoreactivity of GLP-1R on microglia was upregulated after ischemia reperfusion

We previously identified that spinal dorsal horn GLP-1Rs were specifically expressed on spinal microglial cells, and upregulated accompanying microglial proliferation and hypertrophy after peripheral nerve injury [2]. The activation of microglia in response to ischemia/reperfusion was also reported, and the inflammatory response peaked at 48–72 h in the ischemia model [2,36,37]. In this study, we evaluated the ischemia reperfusion-induced changes in GLP-1Rs and microglia in the hippocampus by harvesting brains from six rats 48 h after they were subjected to
transient cerebral ischemia and reperfusion. Hippocampal sections from both the contralateral and ipsilateral sides (Fig. 1a and b) revealed a typical microglial immunostaining pattern (marked by CD11b/OX42), as highly ramified cells spread throughout the hippocampus. Several hippocampal regions could be discerned by their differential immunostaining intensity, i.e., the more intensely stained areas included the stratum lacunosum molecular layer of the CA1 region and the hilus of the dentate gyrus region. In contrast, the stratum pyramidale cell layer was demarcated by its relative lack of immunofluorescence immunostaining. After ischemia–reperfusion, increased microglial immunoreactivity became apparent in the ipsilateral side, and more prominently in the stratum lacunosum molecular layer of the CA1 region. Following ischemia, the microglial immunostaining in the stratum lacunosum molecular layer was upregulated by 60.7%, as measured using the computer-assisted image analysis program ($P<0.05$, paired Student $t$-test) (Fig. 1c). In addition, the expression pattern of GLP-1R immunofluorescence was similar to that of CD11b in both the contralateral and ipsilateral sides (Fig. 1d and e), with an upregulated expression of 48.1% in the ipsilateral side ($P<0.05$, paired Student $t$-test) (Fig. 1f). Finally, the co-localization staining of OX42 and GLP-1R was expressed in both the contralateral and ipsilateral sides (Fig. 1g and h). Ischemia-induced GLP-1R overexpression was seen predominantly in microglia, with little GLP-1R labeling in other cell types. Ischemia upregulated the co-localization immunofluorescence staining of the GLP-1R and microglial marker OX42 in the ipsilateral side by 43.5% compared to the contralateral side ($P<0.05$, paired Student $t$-test) (Fig. 1i), although it is unclear whether GLP-1R induction is primarily due to the induction of microglial cell numbers, or due to its enhanced expression in microglial cells.

3.2. Exenatide and catalpol reduced infarction and improved neurobehavioral manifestation

The neuroprotective effects of exenatide and catalpol were studied in ischemic rats through the intraventricular route as peptidic exenatide is not orally–available and does not readily pass the blood brain barrier although catalpol can be given systemically as an herbal ingredient. Four groups of rats ($n=6$ in each group) received an i.c.v. injection of saline (5 μL), exenatide (300 ng), or catalpol (100 μg) 15 min before occlusion of the left middle cerebral artery, followed by reperfusion operation 1 h later. The doses of exenatide and catalpol were selected from previous studies [23,30]. The rats underwent neurobehavioral testing and infarct area evaluation 2 days after ischemia/reperfusion. Fig. 2a shows photographs of six consecutive slices from one representative brain sample from each group. All of the portions of the brain slices from the sham rat were stained red, whereas a 1-h transient occlusion in the vehicle-treated MCAO rats produced a significant infarction within the left cerebral cortex, indicated by white matter, due to no reaction with TTC. Exenatide and catalpol markedly reduced the white infarct area. The summarized results from the four groups of rats showed that ischemia–reperfusion induced approximately 40% brain infarction in the ipsilateral hemispheres; i.c.v. injection of exenatide and catalpol reduced the infarction by approximately 80% ($P<0.05$, one-way ANOVA followed by post-hoc Student–Newman–Keuls test) (Fig. 2b). Ischemia-reperfusion also had marked effects on the rats' neurobehaviors, reflected by an increase in neurobehavioral scores to 3.0. The i.c.v. injection of exenatide and catalpol slightly but significantly restored neurobehavioral scores by 22–26% ($P<0.05$, one-way ANOVA followed by post-hoc Student–Newman–Keuls test) (Fig. 2c). The infarct area was selected for the later mechanistic studies.
3.3. Exendin (9–39) prevented exenatide and catalpol neuroprotection

Exendin (9–39) is a competitive antagonist that binds the GLP-1R [38,39]. To examine whether exenatide and catalpol produced their neuroprotective effects via the activation of GLP-1Rs, exendin (9–39) was used in the rat transient ischemia/reperfusion model. Six groups of rats (n = 6–8 in each group) received two i.c.v. injections of the control and test substances 15 min before the occlusion of the middle artery, followed by reperfusion 1 h later. The treatments were as follows: 5 μL saline + 5 μL saline, 15 μg exendin (9–39) + 5 μL saline, 5 μL saline + 300 ng exenatide, 5 μL saline + 100 μg catalpol, 15 μg exendin (9–39) + 300 ng exenatide, and 15 μg exendin (9–39) + 100 μg catalpol. The second treatment was administered 15 min after the first one. Fig. 3a shows photographs of six consecutive slices from one representative brain sample from each group. Compared with the saline-treated control rats, i.c.v. injection of exendin (9–39) alone did not have a significant effect on the ischemia reperfusion-induced infarction. However, i.c.v. treatment with exendin and catalpol significantly reduced the infarction by 49.4% and 68.3%, respectively. Pretreatment with exendin (9–39) completely prevented the protective effects of exenatide and catalpol (P < 0.05, one-way ANOVA followed by post-hoc Student–Newman–Keuls test).

3.4. Exenatide and catalpol stimulated β-endorphin expression in the hippocampus and primary cultures of microglia

In our previous study, we showed that exenatide stimulated the expression of β-endorphin in the spinal cord [2]. In this study, we further tested whether exenatide and catalpol stimulated the expression of β-endorphin in the hippocampus. Three groups of rats (n = 6–7 in each group) received an i.c.v. injection of saline (5 μL), exenatide (300 ng), or catalpol (100 μg) 15 min before the occlusion of the middle artery, followed by reperfusion 1 h later. The brain was removed 30 min after reperfusion and ipsilateral hippocampal homogenates were used to measure the β-endorphin level. Data are presented as mean ± SEM (n = 6–7 in each group). * Denotes statistical significance compared with the saline control group (P < 0.05 by one-way ANOVA, followed by post-hoc Student–Newman–Keuls test).

Furthermore, microglial cells originated from the hippocampus were treated with different concentrations (3 × 10−9, 1 × 10−8, 3 × 10−8, 1 × 10−7, and 1 × 10−6 M) of exenatide, or different concentrations (1 × 10−5, 3 × 10−5, 1 × 10−4, 3 × 10−4, 1 × 10−3, and 3 × 10−3 M) of catalpol, and the level of β-endorphin in culture medium were measured 2 h later. Exenatide stimulated the β-endorphin release into the culture medium in a concentration-dependent manner, with an EC50 of 7.6 nM (Fig. 5a). Catalpol also concentration-dependently stimulated β-endorphin release from cultured primary microglia, with an EC50 value of 119 μM (Fig. 5b). In contrast, exenatide at 30 nM (Fig. 5c), or catalpol at 1 mM (Fig. 5d), did not affect β-endorphin secretion from primary cultures of neurons or astrocytes.

3.5. β-Endorphin antiserum and naloxone reversed exenatide and catalpol neuroprotection

To test whether exenatide and catalpol exerted their neuroprotective effects via β-endorphin expression, we used the specific β-endorphin antiserum and opioid receptor antagonist naloxone. Nine groups of rats (n = 8 in each group) received two i.c.v. injections of the control and test substances 15 min before the occlusion of the middle artery, followed by reperfusion 1 h later. The treatments were as follows: 5 μL saline + 5 μL saline, 5 μL β-endorphin antiserum + 5 μL saline, 20 μg naloxone + 5 μL saline + 300 ng exenatide, 5 μL β-endorphin antiserum + 5 μL saline + 100 μg catalpol, and 5 μL β-endorphin antiserum + 5 μL saline + 300 ng exenatide + 100 μg catalpol. The second treatment was administered 15 min after the first one. Exenatide and catalpol significantly reduced the infarction by 49.4% and 68.3%, respectively. Pretreatment with exenatide (9–39) completely prevented the protective effects of exenatide and catalpol (P < 0.05, one-way ANOVA followed by post-hoc Student–Newman–Keuls test).
saline, 5 μL saline + 300 ng exenatide, 5 μL saline + 100 μg catalpol, 5 μL β-endorphin antiserum + 300 ng exenatide, 5 μL β-endorphin antiserum + 100 μg catalpol, 20 μg naloxone + 300 ng exenatide, and 20 μg naloxone + 100 μg catalpol. The second treatment was administered 15 min after the first one. Fig. 6a shows photographs of six consecutive slices from one representative brain sample from each group. Neither i.c.v. injection of the β-endorphin antiserum alone nor naloxone alone significantly altered the ischemia-reperfusion-induced brain infarction compared to the saline control rats, whereas exenatide and catalpol significantly reduced the infarct area by 67.4% and 59.1%, respectively. Pretreatment with the β-endorphin antiserum (without dilution) significantly reversed the protective effects of exenatide and catalpol in infarction by approximately 74% and 77%, respectively. The preliminary results showed that the 1:5 dilution of β-endorphin antiserum was not effective in reducing the protective effect of exenatide or catalpol (data not shown). In addition, pre-treatment with naloxone completely blocked the protective effects of exenatide and catalpol (P<0.05, one-way ANOVA followed by post-hoc Student–Newman–Keuls test) (Fig. 6b).

4. Discussion

Many studies, both in vitro and in vivo, have indicated the therapeutic potential of exenatide in neurodegenerative disorders, and that GLP-1Rs appear to be the key in mediating these effects. Our results showed that in the rat ischemia/reperfusion model, a single lateral ventricular injection of exenatide produced neuroprotection, indicated by a marked reduction in the extent of brain infarction and a mild recovery of neurobehavioral deficits. The results are consistent with the recent findings of several studies, in which exenatide treatment significantly reduced the brain infarct volume or neuronal loss in the CA1 region and improved functional deficits in the transient focal cerebral ischemia models of mice, rats, and gerbils [12,14,40]. Our results further showed that i.c.v. injection of the GLP-1R antagonist exendin (9–39) completely blocked the neuroprotective effect of exenatide, indicating that it is indeed GLP-1R specific. We recently demonstrated that catalpol and other iridoid compounds, geniposide, geniposidic acid, genipin methyl ether, 1,10-anhydrogenipin, loganin, shanzhiside methylester, and 8-O-acetyl shanzhiside methylester, are orthosteric agonists of GLP-1Rs that function similarly in humans and rats and presumably act at the same binding site as exendin (9–39), and that catalpol produced antinociception in formalin-induced tonic hyperalgesia in rats and the antinociceptive effect was blocked by exendin (9–39) [30,41]. In the present study, we further demonstrated that the neuroprotective effect of catalpol was completely blocked by exendin (9–39), suggesting that catalpol, like exenatide, produces neuroprotection via activation of GLP-1Rs in the central nervous system.

As limited access of exenatide to the brain, and as the present study was aimed to be a proof of principle of the GLP-1R/β-endorphin pathway rather than a test of anti-ischemic efficacy, we intraventricularly administered both exenatide and catalpol before the occlusion of the middle cerebral artery. However, extensive preclinical studies have demonstrated that, given systematically after
ischemia was established, exenatide and particularly catalpol were neuroprotective [21,26–28]. Catalpol is a small molecule iridoid glycoside majorly from *R. glutinosa Libosch* and possess an excellent ability to cross the blood–brain barrier [42]. It was reported that intraperitoneal injection of catalpol (1–10 mg/kg) immediately after reperfusion and repeatedly at 12, 24, 48 and 72 h significantly rescued neurons in hippocampal CA1 subfield and reduced working errors during behavioral testing in gerbils subjected to transient global cerebral ischemia. Catalpol neuroprotection was evident when it was applied 3 h post-ischemia, although its efficacy became weak when it was given at 6 h afterward. Of great encouragement was that catalpol neuroprotection could still be seen in a long period of up to 35 days [27]. It was also reported that catalpol (9 mg/kg), administered intraperitoneally 24 h after stroke and then daily for 3 days, significantly facilitated neurological function recovery, reduced infarction volume, and increased cerebral blood flow in ischemic mice [28]. Taken together, all these results support that the potential of systemic GLP-1R agonists in preventing neuronal damage that occurs after ischemic insults.

GLP-1Rs are expressed in the cortex, neocortex, hypothalamus, brainstem, hippocampus, cerebellum, spinal cord, and dorsal root ganglia [2,7–12]. In the hippocampus, GLP-1Rs were reported to be expressed in neurons, particularly pyramidal neurons [13,43,44]. GLP-1 receptor expression was increased one day after transient cerebral ischemia in gerbils, and GLP-1R immunoreactivity was found not only in pyramidal neurons, but also in astrocytes and GABAergic interneurons [40]. We confirmed the ischemia–induced upregulation of GLP-1Rs in the hippocampus particularly in the stratum lacunosum moleculare layer of the CA1 region. In addition, there is cumulative evidence indicating that microglia in the hippocampus are proliferated and activated [36,37,45]. Our results extended this observation by showing that microglial GLP-1Rs were upregulated in addition to the proliferation and activation of microglia following transient ischemia and reperfusion.

**β-Endorphin** is an endogenous opioid peptide neurotransmitter in both the central and peripheral nervous systems, particularly the hypothalamus and pituitary gland, and it specifically activates the opioid receptors located on neurons [46–48]. Our results demonstrated that exenatide and catalpol blocked ischemia-induced cortical and subcortical infarction and stimulated β-endorphin expression in the ipsilateral hippocampus. In addition, pretreatment with exenatide and catalpol concentration-dependently stimulated β-endorphin release from primary cultures of hippocampal microglia but not neurons or astrocytes. Furthermore, the specific β-endorphin antiserum and µ-opioid receptor preferred antagonist naloxone almost completely reversed the neuroprotective effects of exenatide and catalpol. The blockade effect of naloxone is consistent with the previous findings that activation of opioid receptors exerted its neuroprotective effects in vitro and in vivo [49–52], although there is little literature of β-endorphin itself on neuroprotection. All of these results suggest that activation of hippocampal microglial GLP-1R expresses β-endorphin, which passes the microglial neuronal synapses and stimulates the opioid receptors located on the neurons leading to neuroprotection. However, the present study did not provide direct in vivo evidence which showed in response to GLP-1R stimulation with exenatide and catalpol, GLP-1R-expressing microglial cells in the hippocampal region can specifically produce β-endorphin. Further studies are warranted to confirm the β-endorphin origin of hippocampal microglia by using more sensitive and specific in vivo technologies, such as the radioactive in-situ hybridization in the hippocampus, specific depletion of spinal microglia, and conditional knockout of microglial β-endorphin.

It is known that during episodes of hyperglycemia activation of GLP-1R located on pancreas islet β-cells facilitates insulin release to regulate blood sugar [53–57]. Given that activation of GLP-1Rs expresses β-endorphin from microglia in the spinal dorsal horn [2,24] and the hippocampus (the present study), we postulate that in the central nervous system the activation of GLP-1Rs in microglia stimulates β-endorphin expression to produce analgesia and neuroprotection.

**Conflict of interest**

The authors declare no competing financial interests.

**Author Contributions**

Participated in research design: Wang, Jia.
Conducted experiments: Jia, Gong, Li, Zhu.
Performed data analysis: Jia, Gong, Li, Wang.
Wrote or contributed to the writing of the manuscript: Wang, Jia, Gong, Li.
Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81374000) and the Shanghai Industrial Translational Project (No. 15401901300).

References


