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Activation of the PI3K-Akt pathway promotes neuroprotection of the δ-opioid receptor agonist against cerebral ischemia-reperfusion injury in rat models

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ABSTRACT

The central objective was to identify the role of the PI3K-Akt activation pathway on the neuroprotection of δ-opioid receptor agonist (DADLE) against cerebral ischemia-reperfusion (I/R) injury in a rat model. Fifty-five male Sprague-Dawley (SD) rats were included to establish a middle cerebral artery occlusion (MCAO) model which were then divided into the sham, MCAO, LY294002 (MCAO + DADLE + LY294002 [inhibitor of PI3K-Akt pathway]), DADLE (MCAO + DADLE) and DMSO (MCAO + DADLE + DMSO [dimethyl sulphoxide]) groups. The cerebral infarction (CI) volume and nerve cell apoptosis was determined using TTC and TUNEL staining. Quantitative real-time polymerase chain reaction (qRT-PCR), western blotting and immunohistochemistry staining were applied for the expressions of Bad, Bax, Bcl-2 and cleaved caspase-3. The MCAO group showed higher CI volume, nerve cell apoptosis and cleaved caspase-3 expressions than the DADLE and DMSO groups, which were also higher in the LY294002 group than the DADLE group. Compared with the MCAO group, the mRNA and protein expressions of PI3K and Bcl-2, and the protein expressions of p-Akt and p-Bad were elevated, while the mRNA and protein expressions of Bax were decreased in the DADLE and DMSO groups. Decreased mRNA and protein expressions of PI3K and Bcl-2, reduced protein expressions of p-Akt and p-Bad and elevated mRNA and protein expressions of Bax exhibited in the LY294002 group than the DADLE group. These results indicate that activation of PI3K-Akt pathway promotes the neuroprotection of DADLE against cerebral I/R injury in a rat model by decreasing nerve cells apoptosis.

1. Introduction

When drawing parallels between Ischemic cerebrovascular disease and various other forms of cerebrovascular disease, that pose a threat to human health, Ischemic cerebrovascular disease appears to manifest itself with a high incidence, high disability, high recurrence and high mortality rates [1]. Acute ischemic stroke is the leading cause of severe disability in adults, and accounts for a third of the leading causes of death globally [2]. When blood flow is restored, there is a resultant re-establishment of oxygen delivery to the ischemic sections of the brain that will also be re-established and salvage neurons [3].

This being said, reperfusion itself produces an excess amount of reactive oxygen species (ROS) and free radicals, leading to ischemia/reperfusion (I/R) injury [4]. Reported scientific findings have highlighted that excess production of ROS is a critical factor in the occurrence of reperfusion injury [5].

Danhong injection (DH) has demonstrated to have strong amendatory effects on cerebral I/R injury in rats. This is owing to its antioxidant, anticoagulant, antifibrinolytic and antithrombotic ability [6]. Novel therapeutic strategies for ischemic cerebrovascular disease are in urgent need. More recently, neuroprotective therapies have been proposed, although none of them have been

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proved to be clinically tolerated and effective [7]. Furthermore, it has been demonstrated in I/R injury models that neuroprotection exerts its function by mediating the δ-opioid receptor pathway [8].

Activation of δ-opioid receptors is reported to regulate pain and emotional responses, and displays ligand-biased agonism [9]. The δ-opioid receptor agonist, as a new agent of neuroprotection, has significantly important effects on the central nervous system (CNS) [10]. Phosphoinositide-3-kinase (PI3K) has been reported to mediate post-conditioning neuroprotection [11]. The recent findings of a number of targets for protein kinase B (Akt) suggest that Akt is one of the central kinases and prohibits neuronal death by impinging on the cytoplasmic cell death machinery and by regulating nuclear proteins [12]. Interestingly, PI3K-Akt pathway regulates cell survival and growth in response to extracellular signals and mediates survival signals in a wide range of neuronal cell types [13,14]. Previous findings have highlighted that stimulating Akt signaling via activation of Gi/Go-coupled δ-opioid receptors, and regulating PI3K through Src-dependent transactivation of the IGF-I (insulin-like growth factor-I) receptor, has neuroprotective effects [15]. Therefore, we aimed to explore the neuroprotective mechanism of PI3K-Akt pathway against cerebral I/R injury in rat models through regulation of the δ-opioid receptor agonist.

2. Materials and methods

2.1. Ethical statement

This experiment was conducted in accordance with the Institutional Animal Care and Use Committee (IACUC), and was in agreement with the International Code of ethics and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experiment animals and model establishment

Fifty-five male Sprague-Dawley (SD) rats (6–8 weeks old, weighing 260–290 g) were selected (from Hunan SJA Laboratory Animal Co., Ltd) for the establishment of middle cerebral artery occlusion (MCAO) models.

The rats were fed with irregular food, free drinking water at a temperature of 25–26 °C and relative humidity of 60–70%. Beddings were changed twice a week. All animals were fed in a standard feeding room. Each room was consistent regarding feeding, lighting, water, temperature or other conditions. The left cerebral artery was selected as infraction area. With reference to methods of Zea Longa [16], MCAO models were established by suture techniques, as well as the evaluation and selection of models determined by a nerve function score.

Rat models with a score between 1 and 3 were selected for further experiments. The rectal temperature of the rats was maintained at 37 °C by heaters. Rat models were anaesthetized by injecting 10% chloral hydrate (300 mg/kg) to abdominal cavity, and fixed on operating tables in a supine position. Following disinfection of the skin in the vicinity of the operating area, a median incision in the neck was made and, separated to expose the right common carotid artery (CCA) as well as the external carotid artery. The distal internal carotid artery (ICA) of the models was clipped using a bulldog clamp, and the distal CCA and other branches were ligated. The distal part of the CCA bifurcation was pierced by a 1-ml syringe needle, and inserted with a No.3 fishing line of 0.26-mm-diameter from the incision through the ICA to artery origin in brain, with a length of insertion approximately 18–20 mm. Following this, the incision was sutured layer by layer.

2.3. Animal grouping

The fifty-five male SD rats were divided into five groups (11 rats per group), including the sham, MCAO, DADLE (MCAO + DADLE [δ-opioid receptor agonist]), LY294002 (MCAO + DADLE + LY294002 [specific inhibitor of PI3K-Akt pathway]) and DMSO groups (MCAO + DADLE + DMSO [dimethyl sulphoxide]). At the 2 h period of cerebral I/R injury, MCAO models were assessed using the nerve function score, and dead rats or rats with a score over 3 points were eliminated. MCAO model surgery was conducted according to the above methods. In the sham group, the artery was separated without ligation, line insertion or medicine treatment. LY294002 (Cell Signaling Technologies [CST], Beverly, Massachusetts, USA) is an inhibitor of PI3K-Akt pathway, and was diluted with DMSO to 10 mM solution, which (10 μl) was injected to the lateral ventricle half an hour before modeling. DADLE (Sigma-Aldrich Chemical Company, St Louis, MO, USA) is δ-opioid receptor agonist, and 0.5 mg/ml DADLE was injected immediately into the abdominal cavity after model establishment at a dose of 5 ml/kg. The DMSO group was the negative control group of the LY294002 group, where rats were injected into the lateral ventricle with LY294002 (10 μl) half an hour before model establishment.

2.4. Behavioral observation

In accordance with Zea Longa scoring, the nerve function of each rat model was observed and evaluated, nerve function score was recorded by an observer who was blind to the animal grouping system [17], The higher the score was, the more serious the cerebral I/R injury was. Details were as follows: zero point, normal, no neurologic defect; one point, minor neurologic deficit, unable to extend contralateral forepaw, and bodies and forepaws bended to one side when the tails were raised; two points, moderate neurologic deficit, rats were circled to the side of injury, chasing their tails, but presented normal posture when static; three points, severe neurologic deficit, rats fell to the side of injury when static; and four points, very serious neurologic deficit, unable to walk by themselves, and no consciousness was observed.

2.5. Tissue collection

After the 72 h point following cerebral I/R injury, rat models were anaesthetized by injecting 10% chloral hydrate (300 mg/kg) to abdominal cavity. After blood was collected from the aorta of abdomen, anocelia of all rats was opened to expose the heart and the left ventricular was then cut. Pipes were inserted from the left ventricle until the level of aorta was reached. The rats were decapitated and their brain tissues were collected after normal saline was promptly injected for washing. Brain tissues were reserved in a refrigerator at a temperature of −80 °C for 2, 3, 5-triphenyltetrazolium chloride (TTC) staining, western blotting and quantitative real-time polymerase chain reaction (qRT-PCR). The brain tissues were perfusion-fixed using 4% paraformaldehyde for 24 h, soaked in a wax box following dehydration by alcohols, and later completely fixed. The embedded device was put on ice to cool the brain tissues. Coronal sections were obtained from the frozen brain tissues (5 μm) and reserved at 4 °C for transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining and immunohistochemistry staining.

2.6. TTC staining

Fresh brain tissues (kept at −80 °C) were cut into 2 mm pieces at 2 mm distance from forehead along the coronal plane. A ratio of one tissue to 5 pieces was employed. This allowed for observation