Neuroscience Letters 642 (2017) 136–141

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Electroacupuncture preconditioning and postconditioning inhibit apoptosis and neuroinflammation induced by spinal cord ischemia reperfusion injury through enhancing autophagy in rats

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HIGHLIGHTS

- Electroacupuncture preconditioning and postconditioning upregulated autophagy after spinal cord I/R injury.
- Electroacupuncture preconditioning and postconditioning improved neurological function recovery.
- Electroacupuncture preconditioning and postconditioning inhibited apoptosis through enhancing autophagy.
- Electroacupuncture preconditioning and postconditioning inhibited inflammation through enhancing autophagy.

ARTICLE INFO

Article history:
Received 23 July 2016
Received in revised form
11 November 2016
Accepted 6 February 2017
Available online 7 February 2017

Keywords:
Electroacupuncture
Autophagy
Spinal cord ischemia reperfusion injury
Apoptosis
Neuroinflammation

ABSTRACT

Electroacupuncture (EA) has beneficial effects on spinal cord ischemia reperfusion (I/R) injury, but the underlying mechanisms are not fully understood. This study aimed to investigate the role of autophagy in the protection of EA preconditioning and postconditioning against spinal cord I/R injury. For this, spinal cord I/R injury was induced by 14 min occlusion of the aortic arch, and rats were treated with EA for 20 min before or after the surgery. The expression of autophagy components, light chain 3 and Beclin 1, was assessed by Western blot. The hind-limb motor function was assessed using the Basso-Beattie-Bresnahan (BBB) criteria, and motor neurons in the ventral gray matter were counted by histological examination. The apoptosis of neurocyte was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. The expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and matrix metalloproteinase-9 (MMP-9) was also measured using Western blot or enzyme-linked immunosorbent assay (ELISA). Either EA preconditioning or postconditioning enhanced autophagy, and minimized the neuromotor dysfunction and histopathological deficits after spinal cord I/R injury. In addition, EA suppressed I/R-induced apoptosis and increased in the expression of TNF-α, IL-1β, and MMP-9. In contrast, the autophagic inhibitor (3-methyladenine, 3-MA) inhibited the neuroprotective effects of EA. Moreover, 3-MA increased the apoptosis and the expression of TNF-α, IL-1β, and MMP-9. In summary, these findings suggested that EA preconditioning and postconditioning could alleviate spinal cord I/R injury, which was partly mediated by autophagy upregulation-induced inhibition of apoptosis and neuroinflammation.

1. Introduction

Spinal cord ischemia reperfusion (I/R) injury is a major complication of surgery for thoraco-abdominal aneurysms, which may cause a high incidence of paraplegia. Acupuncture is an ancient technique for treating spinal cord injury in traditional Chinese medicine. Previous studies showed that electroacupuncture (EA) contributed toward neurological function recovery in the models of human and animal spinal cord injury [1–6]. Although an increasing data on the neuroprotective effects of EA is available, the ther-

Abbreviations: EA, electroacupuncture; I/R, ischemia reperfusion; BBB, Basso-Beattie-Bresnahan; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MMP-9, matrix metalloproteinase-9; ELISA, enzyme-linked immunosorbent assay; 3-MA, 3-methyladenine; PBS, phosphate buffer saline; DAPI, 4′,6-diamidino-2-phenylindole; LC3, microtubule-associated protein light chain 3; HRP, horseradish peroxidase.

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http://dx.doi.org/10.1016/j.neulet.2017.02.010
0304-3940/

apeutic mechanism remains unclear. The following mechanisms are supposed to be responsible for EA: (1) inhibition of neuronal apoptosis [7,8], (2) promotion of oligodendrocyte proliferation and inhibition of oligodendrocyte death [6], (3) upregulation of neurotrophic factors [9], and (4) antioxidation and anti-inflammation [5].

Autophagy is an intracellular catabolic mechanism that maintains a balance between protein synthesis and degradation. Autophagy has been confirmed to be involved in several central nervous system diseases [10–13], and recently it has aroused increasing interest for its role in spinal cord injury [14–16].

This study used a rat model of spinal cord I/R injury to evaluate the potential protective effects of EA preconditioning and postconditioning, and investigate the role of autophagy on apoptosis and neuroinflammation with EA preconditioning and postconditioning.

2. Materials and methods

2.1. Animals

All experimental procedures and postoperative care were carried out with the approval from the ethics committee of the China Medical University and in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health Publication No. 85-23, National Academy Press, Washington DC, revised 1996). Male Sprague-Dawley rats, weighing 200–250 g, were used in this study. All rats were neurologically intact before anesthesia and were housed in standard cages with free access to food and water. They were separately housed after surgery.

2.2. Experimental protocol

A total of 144 rats were randomly assigned to four groups using a random number table. The sham group (n = 24) and I/R group (n = 24) received aortic arch exposure or cross-clamping for 14 min. The Pre group (n = 24) and Pre + 3-MA group (n = 24) received EA preconditioning, and then underwent a 14-min occlusion of the aortic arch. The Post group (n = 24) and Post + 3-MA group (n = 24) underwent a 14-min occlusion of the aortic arch, and then received EA postconditioning. The rats in the Pre + 3-MA and Post + 3-MA group received intraperitoneal 3-methyladenine (3-MA) at 30 min after reperfusion. However, the rats in the other four groups received an intraperitoneal equivalent volume of phosphate buffered saline (PBS). In each group, six rats were used to evaluate neurological function and histological study, six for Western blotting and enzyme-linked immunosorbent assay (ELISA), six for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay at 24 h after spinal cord I/R injury, and the remaining six were used to evaluate neurological function at 7 d after I/R injury.

2.3. Preparation and treatment of 3-MA

3-MA (Sigma-Aldrich), an autophagic inhibitor, was dissolved in dimethyl sulfoxide (25 mg/ml) and further diluted in PBS for a final dose before the intraperitoneal injection. Rats were injected with 3-MA (2.5 mg/kg) or an equivalent volume of vehicle at 30 min after reperfusion.

2.4. Electroacupuncture preconditioning

EA stimulation was performed at three acupoints in the Governor Vessel, which were Jizhong (GV6), Zhiyang (GV9) and Jiaji (EX-B2) [1,17,18]. The GV6 is located on the posterior midline and below the spinous process of the 11th thoracic vertebra. The GV9 is located on the posterior midline and below the spinous process of the 7th thoracic vertebra. The EX-B2 is bilaterally located on the 3 mm lateral from the posterior midline, below the spinous process of the 2nd lumbar vertebra. Stainless silver needles of 0.3 mm in diameter were inserted into the GV6, GV9 and EX-B2 to a depth of 4 mm.

EA stimulation was carried out using the electro-acupuncture apparatus (Model G 6805-2, Shanghai Medical Electronic Apparatus Company, China) for 20 min. Alternating strings of dense-sparse frequencies (60 Hz for 1.05 s and 2 Hz for 2.85 s alternately) were used, and the intensity was adjusted to induce a slight twitch of the hindlimbs ($\leq$ 1 mA), as described in previous studies [1,3].

2.5. Surgical procedure

The spinal cord I/R model was induced by occluding the aortic arch for 14 min through a left lateral thoracotomy approach, as previously reported [19]. All rats were anesthetized with intraperitoneal injection of 4% sodium pentobarbital at an initial dose of 50 mg/kg. Lung ventilation was achieved using a small animal ventilator (Harvard Apparatus, Hollinston, USA) with endotracheal intubation. Body temperature was continuously monitored with a rectal probe and was maintained at 37.5 ± 0.5 °C with the aid of a heated operating table. Catheters were inserted into the left carotid artery and the tail artery to measure proximal and distal blood pressure, respectively (Spacelabs Medical Inc, Redmond, USA). Rats were placed in the lateral position and a small transverse incision between the 2nd and 3rd ribs was made below the left forelimb and shoulder. After entering the thoracic cavity, the aortic arch was separated and exposed. Under direct visualization, the aortic arch was cross-clamped between the left common carotid artery and the left subclavian artery. Occlusion was confirmed using a laser Doppler blood flow monitor (Moor Instruments, Devon, UK). Ischemia was confirmed as a 90% decrease in flow measured at the femoral artery. The ischemia lasted for 14 min, after which the clamp was removed, followed by 24 h or 7 d of reperfusion. Sham-operated rats underwent the same procedure, but no occlusion of the aortic arch was performed. All rats were allowed to recover in a plastic box at 28 °C for 4 h, and subsequently placed in the cages with free access to food and water.

2.6. Neurological assessment

The motor functions of the rats were assessed at 24 h and 7 d after I/R injury using the Basso-Beattie-Bresnahan (BBB) open-field locomotor scale [20]. The final BBB scores were determined by averaging the values from the three observers in a double-blind manner.

2.7. Histological study

Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin. In cases in which the cytoplasm was diffusely eosinophilic, the large motor neuron cells were considered to be necrotic or dead. When basophilic stippling (containing Nissl substance) was observed, the motor neuron cells were considered to be viable or alive. The intact motor neurons in the ventral gray matter were counted in three sections for each rat by a blinded investigator, and the results were averaged.

2.8. TUNEL assay

A TUNEL assay is the most commonly used technique for examining apoptosis via DNA fragmentation. In situ detection of apoptosis in spinal cords was performed through staining using an in situ Cell Death Detection kit, TMR red (Roche Diagnostics GmbH),