Life Sciences 143 (2015) 114–123

Contents lists available at ScienceDirect

Life Sciences

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

Neuroprotective effect of allicin in a rat model of acute spinal cord injury

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ARTICLE INFO

Article history:
Received 3 June 2015
Received in revised form 12 October 2015
Accepted 2 November 2015
Available online 3 November 2015

Keywords:
Spinal cord injury
Allicin
Apoptosis
Inflammation
Oxidative stress
Nrf2

ABSTRACT

Aims: This study aims to investigate the effect of allicin on motor functions and histopathologic changes after spinal cord injury and the mechanism underlying its neuroprotective effects.
Main methods: The motor function of rats was evaluated with the Basso, Beattie, and Bresna test. Histopathologic changes were evaluated by hematoxylin and eosin and Nissl staining. Spinal cord oxidative stress markers were determined by measuring glutathione and malondialdehyde content and superoxide dismutase activity using commercial kits. Inflammatory factors were determined by measuring tumor necrosis factor-α, interleukin-1β and interleukin-6 using ELISA assay. Apoptosis was examined using TUNEL staining. The effect of allicin on Nrf2 protein levels and localization was assessed using immunofluorescence staining and Western blotting analysis.
Key findings: Results demonstrated that allicin accelerated the motor functional recovery and protected neuron damage against spinal cord injury (SCI). SCI-induced oxidative stress, inflammatory response and cell apoptosis in the spinal cord were also prevented by allicin. In addition, we observed that SCI increased Nrf2 nuclear expression, and allicin treatment further increased Nrf2 nuclear translocation in neurons and astrocytes. siRNA-mediated Nrf2 gene knockdown completely blocked the effect of allicin on spinal cord tissue.
Significance: Our finding suggests that allicin promotes the recovery of motor function after SCI in rats, and this effect may be related to its anti-oxidant, anti-inflammatory and anti-apoptotic effects. Allicin mediated Nrf2 nuclear translocation may be involved in the protective effect as well.

1. Introduction

Acute traumatic spinal cord injury (SCI) usually results in catastrophic neural dysfunction which negatively affects the quality of life of patients. The biphasic injury process in SCI includes primary and secondary injury mechanisms. The primary injury is caused by the initial physical impact, which is characterized by acute bleeding and ischemia. Following the primary insult, several pathways trigger the secondary injury, causing reactive glial changes, neuronal inflammation, oxidative stress, neuronal degeneration and apoptosis [1–4]. Even worse, the spontaneous anatomical and functional recovery will be prevented by these subsequent pathological events. Unfortunately, primary injury caused neuronal loss cannot be restored. Thus, most of the attention has been paid on the development of therapeutic strategy for the secondary injury. However, despite numerous promising experimental studies have been reported, effective treatment that can overcome secondary damage after SCI is lacking.

The nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway is a classical signal which is responsible for the cellular redox homeostasis. The activation of Nrf2/ARE is one of the main defense mechanisms against oxidative stress [5–7] and has been reported to be neuroprotective after SCI [8]. In SCI rats, Nrf2 levels were peaked at 30 min after SCI in cytoplasmic fractions and remained elevated for 3 days, and levels in nuclear fractions elevated at 30 min and peaked at 6 h following SCI. In addition, pharmacological activation of Nrf2/ARE pathway may contribute to the locomotion recovery and inhibit inflammatory response [8,9].

Allicin (diallyl thiosulfinate), is a small molecule extracted from the garlic. It is known as one of the most biologically active compounds in garlic, which is responsible for most of the functions of garlic [10]. Numerous studies have demonstrated the pharmacological effect of allicin, including anti-inflammatory, antimicrobial, antifungal, antiparasitic, antihypertensive, anti-diabetic and anti-tumor activities [11,12]. Recent in vitro and in vivo studies reported that allicin could prevent traumatic neuronal injury in rat cortical neurons and traumatic brain injury rats by attenuating oxidative stress, inhibiting inflammatory response and neuron apoptosis [13,14]. In addition, allicin has been found to have neuroprotective effect against spinal cord ischemia/reperfusion injury in rabbits [15]. However, its effect on acute traumatic spinal cord injury

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http://dx.doi.org/10.1016/j.lfs.2015.11.001
0024-3205/

has not been investigated. In addition, Nrf2 activation has been approved to be beneficial in the recovery of SCI [16,17]. In a previous study, allicin has been found to active Nrf2 signaling pathway in cardiac hypertrophy rats [18]. Therefore, we hypothesize that allicin would have neuroprotective effect via Nrf2 activation and aim to prove it in this study.

2. Material and methods

2.1. Establishment of animal models of spinal cord injury

A total of 90 female Sprague–Dawley rats weighing 200–250 g were purchased from Shanghai SLAC Laboratory Animal Co (Shanghai, China). All the animal experiments were performed in accordance with the guidelines of the Ethical Committee of Experimental Animals of Second Military Medical University. After being acclimatized for 2 weeks, rats were randomly divided into the following groups (n = 18): (1) sham; (2) SCI; (3) SCI + allicin 2 mg/kg (allicin L); (4) SCI + allicin 10 mg/kg (allicin M); (5) SCI + allicin 50 mg/kg (allicin H). All the rats were intraperitoneally anesthetized with 30 mg/kg pentobarbital sodium. The lower back was shaved and sterilized. A median incision was made on the back taking T8–9 spinous process as a center to expose T7–10 spinous processes and the lamina and a laminectomy was performed at the T8 level. Rats in the sham group received only T8 laminectomy. Rats in the SCI groups received an injury in accordance with Allen's method. Briefly, a self-made 10 g rod was dropped vertically from a height of 50 mm on the exposed spinal cord. The rod was rest on the injury site for 3 min. The wound was then washed and the tissue was sutured. NaCl (5 mL of 0.9%) was injected intraperitoneally immediately after surgery, and penicillin (200,000 units/day, intramuscular injection) was used to protect from postoperative infection. Urinary bladder was manually emptied twice daily to assist in urination until the micturition function returned to normal. Rats in allicin groups received an intraperitoneally injection of allicin (MB5783, Meilun, Dalian, China) one hour before the surgery and once daily for 21 days. Rats in the sham and SCI groups received 0.9% NaCl daily.

2.2. siRNA design and delivery

siRNAs encoding Nrf2 were purchased from GenePharma (Shanghai, China). Sequence of the siRNA used in the present study is as followed: CCGGAGAAAUUCCUCCCAAUTT. The negative scrambled sequence is as follows: UUCUCCGAACGUGUCACGUTT. After surgery, a hole was created at the exposed dura using a hypodermic syringe. An intrathecal catheter filled with sterile PBS was inserted 7 mm cephalad into the dura. The catheter exposed epidural was fixed tightly on the surrounding soft tissue. The end of the catheter was fixed on the skin and was sealed sterilely. Rats in siRNA treated groups received daily 5 μg/5 μl of Nrf2-siRNA or scramble siRNA through the intrathecal catheter for 3 days successively. The mRNA and protein expression of Nrf2 in the spinal cord was measured by real-time PCR and Western blot, respectively.

2.3. Evaluation of motor function

Six rats in each group were randomly selected to perform motor function test at day 1, 4, 7, 14 and 21 after the surgery. Motor function was assessed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale [19]. The range of BBB locomotor scale scores was between 0 and 21. Twenty-one points refer to normal motor capacity and lower scores indicate impairment in motor capacity. Each rat was evaluated three times by three observers who were blinded to the treatment group, and the mean of the three measurements was taken.

2.4. Histological examinations

After the 21-day treatment, six rats in each group were deeply anesthetized and perfused with 4% paraformaldehyde via the left ventricle for pre-fix. A 1.5 cm spinal cord tissue surrounding the damage site was collected and post-fixed in 4% paraformaldehyde overnight. Fixed tissues were dehydrated using a series of ethanol, embedded in paraffin, and serially sectioned into 4 μm thick coronal slices. To perform staining, sections were deparaffinized in xylene and hydrated using a series of ethanol. Three sections of each group were used for hematoxylin and eosin (HE) (Solarbio Science & Technology, Beijing, China) staining. Three sections were stained with thionine (Solarbio). Five fields within each slide were randomly selected for neuronal counts. Sections were viewed under an optical microscope (DP73; Olympus, Tokyo, Japan). The cavity area was measured on sections stained with HE using IMAGE J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Tissue preparation and protein quantification

After the 21-day treatment, the remained 6 rats in each group were deeply anesthetized and spinal cord tissue surrounding the damage site was immediately collected. Tissues were homogenized by a homogenizer in cooled RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) on ice. Homogenates were then centrifuged at 15,000 g for 10 min at 4 °C. Nuclear and cytosolic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) following the manufacturer's instruction. The protein concentrations of the supernatants were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime).

2.6. Biochemical determination

Spinal cord tissues were homogenized in cooled PBS and repeated freezing in liquid nitrogen and thawing for three times. Homogenates were then centrifuged at 15,000 g for 10 min at 4 °C. Supernatants were used for the measurement of MDA and GSH levels and determination of SOD activity according to the protocols in the commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Spinal cord protein supernatants were prepared in RIPA as described below. The determination of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were performed using commercially available ELISA kits special for rats (USCN Life Science, Wuhan, China) according to the manufacturer's instructions. Concentrations are given in pg/mg protein.

2.8. Real-time quantitative PCR

Total RNA from tissue specimens in each group were purified using an RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. Oligonucleotide primer and super Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (BioTeke, Beijing, China) were used to make cDNA in a 20-μL reaction mixture. Quantitative real-time PCR was performed on 1-μL cDNA using 10-μL SYBR-Green Master Mix (Tiangen Biotechnology Co., Ltd., Beijing, China), 1-μL cDNA and 10 μM of forward and reverse primers on an Exicycler™ 96 real-time quantitative thermal block (Bioneer, Daejeon, Korea) in a 20-μL reaction mixture. The sequences of primers are as follows: HO-1, forward: 5′- CTGGAATGGAAGGAGATGCC-3′, reverse: 5′- TCAGAACAGCCGCCTCTACCG-3′, and β-actin, forward: 5′- GGAGAT TACTGCCCTGGCTCCTAGC-3′, reverse: 5′- GGCCGGACTCATCGTACTCC TGCTT-3′ (Sangon Biotech, Shanghai, China). The reaction was performed in an Exicycler™ 96 real-time quantitative thermal block (Bioneer, Taejon, Korea). Data were analyzed by using the $2^{-\Delta\Delta Ct}$ method.