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Neuroprotective effect of allicin against traumatic brain injury via Akt/endothelial nitric oxide synthase pathway-mediated anti-inflammatory and anti-oxidative activities

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ABSTRACT

Allicin, one of the main biologically active compounds derived from garlic, has been shown to exert various anti-oxidative and anti-inflammatory activities in in vitro and in vivo studies. Here, we sought to investigate the potential neuroprotective effects of allicin against traumatic brain injury (TBI) in rats. We found that allicin treatment (10 and 50 mg/kg, not 1 mg/kg) significantly reduced brain edema and motor functional deficits, as well as apoptotic neuronal cell death in injured cortex. These protective effects could be observed even if the administration was delayed to 4 h after injury. Moreover, allicin treatment decreased the expression levels of MDA and protein carbonyl, preserved the endogenous antioxidant enzyme activities, and suppressed the expression of inflammatory cytokines. The results of Western blot analysis showed that allicin increased the phosphorylation of Akt and endothelial nitric oxide synthase (eNOS). Blocking Akt/eNOS pathway activation by specific inhibitor LY294002 (10 μL, 10 mmol/L) or L-NIO (0.5 mg/kg) partly reversed the protective effects of allicin and its anti-inflammatory activities. The allicin induced anti-oxidative activity was partly prevented by LY294002, but not L-NIO. In summary, our data strongly suggested that allicin treatment at an appropriate dose can exert protective effect against TBI through Akt/eNOS pathway-mediated anti-inflammatory and anti-oxidative activities.

1. Introduction

Traumatic brain injury (TBI) is defined as damage to the brain resulting from external mechanical force, including accelerating, decelerating and rotating forces (Bayston et al., 2000). TBI is a leading cause of death and disability around the world and presents a major worldwide social, economic and health problem. Although TBI induced death has declined due to the improvement of treatment and increased societies wealthy to provide modern emergency and neurosurgical services, the average mortality rate is estimated to be 21% by 30 days after TBI in the United States (Greenwald et al., 2003). In recent years, great effects have been made to investigate the molecular mechanisms underlying neuronal injury after TBI, while clinical trials to test agents that could halt these cellular mechanisms have met largely with failure (Maas et al., 2008).

It is well known that a large percentage of people killed by TBI do not die at the moment of injury but rather days to weeks after the event. Thus, brain damage after TBI can be classified by its time course into primary injury and secondary injury, which is considered to be the reason why 40% of TBI patients deteriorate even after being hospitalized (Narayan et al., 2002). Secondary injury occurs in hours or days after the injury as a consequence of systemic or intracranial complications, such as damage of the blood–brain barrier, release of inflammatory factors, free radical overload, excessive release of excitatory neurotransmitters, influx of calcium and sodium ions into neuronal cells, as well as dysfunction of mitochondria (Chen et al., 2012b, 2013a,b). The gradual processes of secondary injury may provide doctors with so-called "golden hour" for pharmacological intervention, and a lot of agents and compounds targeting secondary brain injury factors have been shown to be protective in experimental TBI models (Park et al., 2008).

Garlic (*Allium sativum* L.) has been recognized as a valuable folk medicine for many centuries, and modern scientific research has shown that a vast spectrum of health beneficial effects were attributed to garlic, including anti-inflammatory, anti-microbial, anti-fungal, anti-parasitic, anti-hypertensive and anti-cancer activities

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(Bolton et al., 1982; Chung, 2006). One of the main biologically active compounds derived from garlic, allicin (diallyl thiosulfinate), which formed by the interaction of the enzyme alliinase with its substrate alliin, is responsible for most of functions of garlic (Zhu et al., 2012). Previous reports have shown that allicin is effective in preserving cardiac function via attenuating reactive oxygen species (ROS)-dependent signaling pathways (Liu et al., 2010). Allicin was also demonstrated to exert neuroprotective effects through reducing neuronal death and ameliorating the spatial memory impairment in Alzheimer's disease models (Li et al., 2010). However, there are no studies to date of the effect of allicin on TBI. In the present study, we investigate the potential neuroprotective efficacy of allicin as well as the relationship between allicin induced neuroprotection and Akt/endothelial nitric oxide synthase (eNOS) pathways in a model of TBI in rats.

2. Materials and methods

2.1. Traumatic brain injury

TBI was induced by using a controlled cortical impact (CCI) model in according with previously detailed methods (Chen et al., 2011). Briefly, rats were anaesthetized with an intraperitoneally administered sodium pentobarbital (50 mg/kg) and placed in the stereotaxic frame. A unilateral circular craniotomy (7 mm), centred 3.5 mm posterior and 4 mm lateral to bregma was performed by a portable drill after the midline incision. To induce injury, a pneumatic piston impactor device with a 3 mm diameter and rounded tip was used to impact the brain at a depth of 1 mm (velocity 5 m/s). During surgery, a warming pad with feedback temperature control ensured a sustained normal body temperature.

2.2. Experimental design

Experimental 1: Effects of allicin on neuronal damage after TBI. We randomly divided 120 rats into five groups: sham, vehicle and three allicin treated groups, which were treated with allicin at the concentration of 1, 10 or 50 mg/kg (IP) immediately after TBI. The animals in sham group were only subjected to surgical procedures, while other animals were subjected to traumatic injury. The vehicle group rats were intraperitoneally administered with an equal volume of the solution used to dissolve allicin at the same time. Thirty rats were used to evaluate brain water content at 24 h after injury (n = 6/group); thirty rats were used to TUNEL staining at 24 h after injury (n = 6/group); thirty rats were used to Western blot analysis at 24 h after injury (n = 6/group); the remaining 30 rats were used to assess neurological function up to 14 days after injury and contusion volume at 14 and 28 days after injury (n = 6/group).

Experimental 2: Therapeutic window of allicin against TBI. We randomly divided 90 rats into five groups: vehicle and four allicin treated groups, which were administrated with 50 mg/kg allicin at 0, 2, 4 or 8 h after TBI, respectively. The vehicle group rats were intraperitoneally administered with an equal volume of the solution used to dissolve allicin. Thirty rats were used to evaluate brain water content at 24 h after injury (n = 6/group); thirty rats were used to TUNEL staining at 24 h after injury (n = 6/group); the remaining 30 rats were used to assess neurological function at 7 and 14 days after injury (n = 6/group).

Experimental 3: Effects of allicin on TBI induced oxidative stress and inflammatory cytokines. We used an additional 36 rats in this experiment and divided then into three groups: sham, vehicle and allicin treated group, which was administrated with 50 mg/kg allicin immediately after TBI. Eighteen rats were used to detect the antioxidative enzymes, oxidative products and inflammatory cytokines at 24 h after injury (n = 6/group). The remaining 18 animals were used to measure the expression of inflammatory cytokines at 6 h after TBI (n = 6/group).

Experimental 4: Involvement of Atk and eNOS in allicin induced neuroprotection. We used an additional 150 rats in this experiment and divided then into five groups: sham, vehicle, allicin, allicin + LY and allicin L-NIO. The last two groups were pretreated with LY294002 (LY, 10 μL, 10 mmol/L) or L-NIO (0.5 mg/kg) at 30 min before TBI. Every 30 animals were used to detect brain water content, neuronal apoptosis, neurological deficit scores, inflammatory cytokines, oxidative products and antioxidative enzymes, respectively (n = 6/group).

2.3. Evaluation of brain edema

Brain edema was determined by measuring brain water content with the wet–dry method at 24 h after TBI. After rats were anesthetized and sacrificed by decapitation, the brains were quickly removed and separated through the interhemispheric fistula into left and right hemispheres. Tissue samples from injured hemispheres were weighed immediately to get wet weight. After drying in an oven for 48 h at 100 °C, the tissues were reweighed to yield the dry weight. Brain water content was then calculated using the following formula: $\%\ H_2O = (1 - \text{dry weight/wet weight}) \times 100\%$.

2.4. Assessment of contusion volume

Contusion volume and brain tissue loss were measured by morphometric image analysis. After rats were anesthetized and sacrificed by decapitation, the brains were quickly removed. At each 500 μm interval, 30 μm sections were mounted on slides and stained with 0.2% cresyl violet solution for contusion volume calculation. The areas of the lesions were integrated, and the results were represented as a volume percentage of the lesion compared with the contralateral hemisphere to avoid the interference from brain edema in the ipsilateral hemisphere.

2.5. Measurement of neurological deficit

The prehensile traction and beam-balancing tests were used for neurological evaluation as described previously (Dixon et al., 1987; He et al., 2012). The scoring of prehensile traction test was as follows: 0 = rat grasps the string tightly and climbs up quickly; 1 = rat holds onto the string with its rear limbs and tries to climb beyond 60 s; 2 = rat remains on the string but does not rely on its rear limbs beyond 60 s; 3 = rat falls down from the string during the period of 30–60 s; 4 = rat falls down from the string within 30 s. The scoring of beam-balancing test was as follows: 0 = rat can walk easily and turns around freely; 1 = rat can maintain a stable posture during 60 s; 2 = rat hugs the beam or hooks the wood with its limb beyond 60 s; 3 = rat falls down from the beam during the period of 30–60 s; 4 = rat falls down from the beam within 30 s.

2.6. TUNEL staining

Neuronal apoptosis was measured by TUNEL staining, a method used to observe DNA strand breaks in nuclei. In brief, sections of 4 μm thick were cut and mounted on poly-L-lysine-coated slides, and treated with proteinase K solution (20 μg/mL) for 10 min at room temperature to permeabilize tissues. TUNEL staining was performed by labeling with fluorescein TUNEL reagent mixture for 60 min at 37 °C according to the manufacturer's suggested protocol, and examined under a fluorescence microscopy. The number of TUNEL-positive cells in each section in 10 microscopic fields (at