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Allicin protects rat cortical neurons against mechanical trauma injury by regulating nitric oxide synthase pathways

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ABSTRACT

Allicin, a small molecule that is responsible for the typical smell and most of the functions of garlic, possesses a broad spectrum of pharmacological activities and is considered to have therapeutic potential in many pathologic conditions. In the present study, we investigated the potential protective effect of allicin in an in vitro model of traumatic brain injury (TBI) using primary cultured rat cortical neurons. We found that allicin treatment significantly reduced mechanical trauma-induced lactate dehydrogenase (LDH) release and inhibited apoptotic neuronal death in a dose-dependent manner. These protective effects were observed even if allicin treatment was delayed to 2 h after injury. Allicin significantly decreased the expression of inducible nitric oxide synthase (iNOS) and increased the phosphorylation of endothelial NOS (eNOS) but had no effect on neuronal NOS (nNOS) expression. Allicin-induced protection in cortical neurons was augmented by iNOS and nNOS antagonists and was partly reversed by blocking eNOS phosphorylation. In addition, allicin treatment inhibited the TBI-induced activation of ERK and further enhanced the phosphorylation of Akt in TBI-injured neurons. The Akt inhibitor LY294002 attenuated the allicin-induced increase in eNOS expression and phosphorylation, whereas the ERK inhibitor PD98059 had opposite effects on the expression of iNOS and eNOS. Pretreatment with LY294002 or PD98059 partly prevented or further enhanced allicin-induced neuroprotection, respectively. Collectively, these data demonstrate that allicin treatment may be an effective therapeutic strategy for traumatic neuronal injury and that the potential underlying mechanism involves Akt- and ERK-mediated regulation of NOS pathways.

1. Introduction

Traumatic brain injury (TBI), also known as brain trauma, is defined as a blow or jolt to the head or a penetrating head injury that disrupt the function of the brain. TBI can be classified based on severity (mild, moderate or severe), mechanism (closed or penetrating head injury) or other features (occurring in a specific location or over a widespread area) (Hukkelhoven et al., 2003; Topolovec-Vranic et al., 2012). TBI is a major cause of injury-related hospitalization, disability and death worldwide, especially in children and young adults. According to the literature, an average of 634 000 incidents of TBI, either alone or in conjunction with other injuries, occur each year among children in the United States (Langlois et al., 2006). In spite of dramatic improvements in the management of TBI, the ability to improve patient outcomes is still inadequate (Andriessen et al., 2010).

Garlic, also known as *Allium sativum*, is widely used around the world for its pungent flavor as a seasoning or condiment. Animal studies and clinical trials in humans have suggested the possible medicinal use and health benefits of garlic, which has been used for thousands of years in traditional Chinese medicine for treating various ailments (Ginter and Simko, 2010). Allicin (diallyl thiosulfinate), one of the most biologically active compounds in garlic, is produced during the crushing of garlic cloves and is responsible for the typical smell and most of the functions of garlic (Lawson and Gardner, 2005). Previous in vitro and in vivo studies have shown that allicin possess a variety of biological effects, such as anti-inflammatory, antimicrobial, antifungal, antiparasitic, antihypertensive and anticancer activities (Hunter et al., 2005). An in vitro study indicated that allicin decreases ROS generation and increases the level of glutathione through its antioxidative ability in endothelial cells (Horev-Azaria et al., 2009). A recent study also demonstrated that allicin attenuates spinal cord

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ischemia–reperfusion injury via an improvement of mitochondrial function (Zhu et al., 2012). Based on the above observations, we hypothesized that allicin may have neuroprotective effects against mechanical trauma-induced neuronal injury in cultured rat cortical neurons. In the present study, mechanical trauma injury in cultured cortical neurons, a standard in vitro model of TBI, was used to investigate the potential protective effect of allicin with a focus on nitric oxide synthase pathways.

2. Materials and methods

2.1. Subjects

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to minimize animal number and suffering. Allicin (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Cortical cell culture

Cortical neurons were cultured from Sprague-Dawley rats using a method reported previously (Chen et al., 2012). Briefly, pregnant rats were sacrificed and embryonic day 16–18 cortices were removed. The isolated cortices were dissociated via mechanical trituration and suspended in plating medium containing Neurobasal medium, 2% B27 supplement and 0.5 mM L-glutamine. The culture plates were coated with poly-D-lysine (PLL, 50 μg/ml) at room temperature overnight, and neurons were plated at a density of 3×10^5 cells/cm^2. Glial growth was suppressed by treatment with 5-fluoro-2-deoxyuridine and uridine on the third day after plating. Neurons were maintained at 37 °C in a humidified 5% CO_2 incubator, and half of the culture medium was changed every other day.

2.3. In vitro TBI model

To mimic TBI in vitro, a mechanical trauma injury model in primary cultured cortical neurons was employed as previously described (Mori et al., 2002). Briefly, mechanical injury was induced using a sterile 21-gauge needle to draw parallel scratches across the circular wells of culture plates (9×9 scratches in six-well plates and 6×6 scratches in 24-well plates). These scratches caused immediate neuronal death under the blade followed by a secondary insult to other cells.

2.4. LDH assay

Neuronal injury was quantitatively assessed by measuring the release of LDH, a cytoplasmic enzyme released from injured cells after injury. LDH release into the culture medium was detected using a diagnostic kit according to the manufacturer's instructions (Chen et al., 2011). The results were normalized to the maximal LDH release, which was determined by treating control wells for 60 min with 1% Triton X-100 to lyse all cells.

2.5. TUNEL staining

To investigate the effect of allicin on traumatic neuronal injury induced apoptosis, TUNEL staining was performed using an in situ cell death detection kit. Briefly, cortical neurons were seeded on PLL-coated glass slides at a density of 3×10^5 cells/cm^2, and were subjected to mechanical trauma and various treatments. Then, the neurons were fixed by immersing slides in freshly prepared 4% methanol-free formaldehyde solution in PBS for 20 min at room temperature. The neurons were then permeabilized with 0.2% Triton X-100 for 5 min. Cells were labeled with fluorescein TUNEL reagent mixture for 60 min at 37 °C according to the manufacturer's suggested protocol. Subsequently, the slides were examined by fluorescence microscopy and the number of TUNEL-positive (apoptotic) cells was counted. Hoechst 33342 (10 μg/ml) was used to stain the nucleus.

2.6. Western blot analysis

Cultured cortical neurons were washed with ice-cold PBS, and harvested in lysis buffer containing protease inhibitors. The protein content was determined using a BCA protein assay kit. An amount of 40 μg protein was resolved on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk and incubated with the following primary antibodies: cleaved caspase-3, iNOS, p-eNOS, eNOS, nNOS, p-Akt, Akt, p-ERK, ERK and β-actin. Membranes were then washed and incubated for 1 h at room temperature with secondary antibodies. The Image J analysis software was used to quantify the optical density of each band.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS 16.0 software package. Statistical evaluation of the data was performed

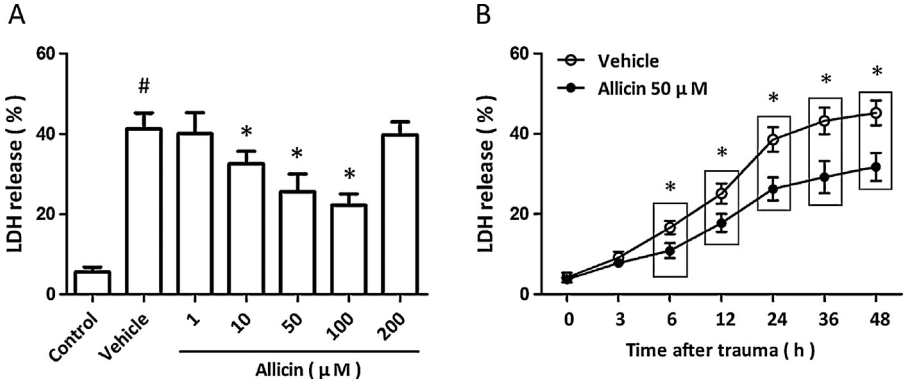

Fig. 1. Allicin attenuates mechanical trauma-induced neuronal injury. Cultured cortical neurons were pretreated with allicin at different concentrations (1 μM, 10 μM, 50 μM, 100 μM and 200 μM) 1 h before injury, and LDH release was assayed 24 h later (A). After pretreatment with 50 μM allicin 1 h before injury, LDH release was assayed at 3 h, 6 h, 12 h, 24 h, 36 h and 48 h after injury (B). The data are represented as the means ± SD from five experiments. $^{\#}P < 0.05$ vs. the control group and $^{*}P < 0.05$ vs. the vehicle group.