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Post-injury administration of allicin attenuates ischemic brain injury through sphingosine kinase 2: *In vivo* and *in vitro* studies

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ABSTRACT

Allicin, one of the main biologically active compounds derived from garlic, has been shown to exert various pharmacological activities and is considered to have therapeutic potential for many pathologic conditions. In the present study, we investigated the potential post-ischemic neuroprotective effects of allicin and its underlying mechanisms. Using a rat middle cerebral artery occlusion (MCAO) model, we found that intraperitoneal treatment with 50 mg/kg allicin significantly reduced brain infarct volume, attenuated cerebral edema and decreased the neurological deficit score. Allicin treatment also diminished TUNEL positive cells and inhibited the activation of caspase-3 after MCAO. These protective effects could be observed even if the administration was delayed to 6 h after injury. In addition, we evaluated the *in vitro* protective effects of allicin against oxygen glucose deprivation (OGD) induced neuronal injury in primary cultured cortical neurons. Allicin (50 μM) increased neuronal viability, decreased lactate dehydrogenase (LDH) release and inhibited apoptotic neuronal death after OGD. These protective effects could be observed even if the administration was delayed to 4 h after injury. Furthermore, allicin significantly increased the expression of sphingosine kinases 2 (Sphk2) both *in vivo* and *in vitro*. Pretreatment with the Sphk2 inhibitor ABC294640 partially reversed the protective effects of allicin against MCAO and OGD injury, indicating that an Sphk2-mediated mechanism was involved in allicin-induced protection in our models. The combination of findings suggests that post-injury administration of allicin has potential as a neuroprotective strategy for ischemic stroke.

1. Introduction

Ischemic stroke is a major cause of morbidity and mortality in the world, and according to data from the American Heart Association, the overall stroke prevalence has not changed over the past decade (Go et al., 2014). Without effective intervention, the number of deaths globally is projected to rise to 6.5 million in 2015 and to 7.8 million in 2030 (Strong et al., 2007). Therefore, there is an urgent need to develop effective treatments for ischemic stroke.

Allium sativum, usually known as garlic, is a common food and has long been valued in Asian folk medicine (Bolton et al., 1982). Allicin (diallyl thiosulfinate, chemical structure shown in Fig. 1) is a major component of garlic and a precursor of many secondary products formed in aged garlic and crushed garlic preparations. It is formed during the chopping, crushing or chewing of garlic cloves through a chemical interaction between alliin, a sulfur-containing amino acid, and the enzyme allicinase (Lawson and Gardner, 2005). Allicin has been shown to exert a variety of beneficial effects, such as anti-inflammatory, anti-microbial, anti-fungal, anti-parasitic, anti-hypertensive and anti-cancer activities (Borlinghaus et al., 2014). Allicin could protect cells against oxidative stress by inducing the generation of antioxidant products, thereby reducing cytotoxic substances and scavenging free radicals (Chan et al., 2013). More recently, allicin was shown to exert neuroprotective activity against traumatic or ischemic neuronal injury by regulating oxidative stress and apoptosis-related cascades (Chen et al., 2014; Liu et al., 2015; Zhou et al., 2014). However, in the vast majority

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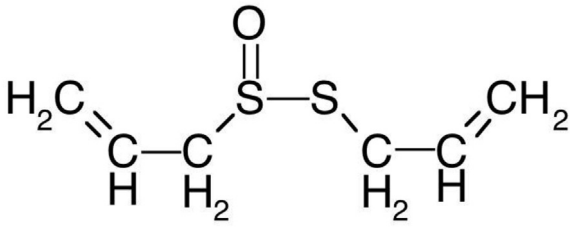

Fig. 1. Chemical structure of allicin.

of previous experiments, allicin was administered prior to injury, which might be problematic in clinical trials because of the difficulty in obtaining informed consent.

Various signaling transduction pathways are triggered in response to cerebral ischemia. Sphingosine 1-phosphate (S1P), one of the sphingolipid metabolites, has recently been shown to function as an important bioactive molecule that controls many cellular processes, such as proliferation and survival (Okada et al., 2009). Sphingosine kinases 1 and 2 (Sphk1 and Sphk2) convert S1P and are the rate-limiting enzymes in the process of endogenous S1P generation (Bryan et al., 2008). Due to their neuron-specific functions, such as the regulation of neurotransmitter release, these two Sphk isoforms are considered to play important roles in several neurological disorders (Okada et al., 2009). The expression of Sphk2, but not of Sphk1, is enhanced by short-duration ischemia that induces preconditioning protection (Wacker et al., 2009). Consequently, the induction of Sphk2 by neuroprotective strategy acts, through neuronal and microvascular mechanisms, to mediate ischemic tolerance (Wacker et al., 2012; Yung et al., 2012). More recently, Sphk1 was shown to mediate neuroinflammation following cerebral ischemia.

Considering the pharmacological properties of allicin and previous studies on the subject, in the present study, we asked whether allicin has protective effects against ischemic brain injury *in vivo* and *in vitro*, and whether post-injury administration of allicin could be beneficial in brain ischemia models. In addition, we also investigated the potential underlying mechanisms with a focus on sphingosine kinases (Sphks).

2. Materials and methods

2.1. In vivo model of brain ischemia

Adult male SD rats weighing 280–300 g were obtained from the Laboratory Animal Center of the Fourth Military Medical University (FMMU). All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the FMMU. Middle cerebral artery occlusion (MCAO) was used to induce focal cerebral ischemia according to previously published methods (Chao et al., 2010). Briefly, rats were anesthetized using 2% isoflurane in oxygen and placed in a stereotaxic frame. Ischemia was produced by advancing the tip of a rounded 0.32 mm nylon suture into the right internal carotid artery through the right external carotid artery. After 60 min of occlusion, the thread was withdrawn to allow reperfusion. The animal's core body temperature was maintained at 37 ± 0.5 °C with a thermostatically controlled heating pad during surgery. Sham-operated animals received midline neck incisions. The right common carotid artery was isolated, but not cut. In allicin-treated groups, animals were intraperitoneally treated with 50 mg/kg allicin at different time points (3, 6 or 9 h) after MCAO. The vehicle group rats were intraperitoneally administered with an equal volume of the solution used to dissolve allicin.

2.2. Measurement of brain edema

The wet–dry method was used to determine brain edema after ischemia. Rats were killed by decapitation under deep anesthesia, and the brain was quickly removed. Tissue samples from the injured hemispheres were dissected and weighed immediately to obtain the wet weight. The dry weight was determined after heating the tissue for 48 h at 100 °C. Brain water content was then calculated using the following formula: $\%H_2O = (1 - \text{dry weight}/\text{wet weight}) \times 100\%$.

2.3. Assessment of neurological deficit score

Neurological deficit scores were measured according to Bederson's method (Bederson et al., 1986). Five categories of motor neurological function were scored: 0: no observable neurological deficits, 1: torso turning to the contra lateral side when lifted by the tail, 2: decreased resistance to lateral push, 3: circling or walking to the right side, 4: unable to walk spontaneously and a depressed level of consciousness.

2.4. Evaluation of infarct volumes

Brain infarct area was evaluated using 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. After obtaining the neurological deficit score, the rats were killed and the brains were sectioned into 2 mm-thick coronal slices. Coronal brain slices were stained in 2% TTC at 37 °C for 15 min in the dark and then photographed. The infarct tissue areas were measured using Image-Pro Plus software. To account for edema, the infarcted area was estimated by subtracting the uninfarcted region in the ipsilateral hemisphere from the contralateral hemisphere, and the infarct volume was expressed as a percentage of the contralateral hemisphere.

2.5. TUNEL staining

Neuronal apoptosis was measured by TUNEL staining. In brief, brain sections of 4 μm thick were cut and treated with proteinase K solution (20 μg/ml) for 10 min at room temperature to permeabilize the tissues. TUNEL staining was performed by labeling the tissues with a fluorescein TUNEL reagent (Red) mixture for 60 min at 37 °C according to the manufacturer's suggested protocol, and the tissues were examined under a fluorescence microscopy. The number of TUNEL-positive cells was counted by an investigator blinded to the grouping.

2.6. Measurement of caspase-3 activity

The activity of caspase-3 was measured using a colorimetric assay kit according to the manufacturer's instructions (Cell Signaling, MA, USA). Briefly, cell and tissue homogenates were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate, and the absorbance at 405 nm was measured after incubation at 37 °C for 4 h. Absorbance of each sample was determined by subtracting the mean absorbance of the blank and corrected by the protein concentration of the cell lysate. The results were described as relative activity compared to the control group.

2.7. Primary cultures of cortical neurons

Cortical neurons were cultured from SD rats according to previously published methods (Chen et al., 2012). Briefly, cerebral