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Original Article

Allicin attenuates global cerebral ischemia/reperfusion injury in gerbils via anti-oxidative and anti-apoptotic pathway

Juan Yu[1*], Ru-Xia Liu[1*], Peng Wang[1], Yong-Gang Cao[2], Qian-Long Zhang[1], Hui Qiao[1,3], Qian Luo[1], Shu-Zhi Li[2], Chang-Lin Jiang[4], Li-Hui Qu[1]

Departments of [1]Physiology, [2]Pharmacology, College of Basic Medical Sciences, Harbin Medical University-Daqing, Daqing, China; Departments of [3]Gastroenterology, [4]Pain, Daqing Oilfield General Hospital, Daqing, China. [*]Equal contributors.

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Abstract: Objective: Allicin, the main component which is responsible for the biological activities of garlic, exerts a broad spectrum of pharmacological activities and is regarded as a potential drug in clinical therapy. However, the mechanisms of involvement of allicin in cerebral impairment induced by global cerebral ischemia/reperfusion (I/R) remain unclear. Therefore, here, we explored the effects of allicin on global cerebral I/R injury in gerbils. Methods: 45 gerbils were divided into 5 groups and treated with sham operation or transient global cerebral I/R for 7 min and then intragastric treated with allicin (20, 40, 80 mg/kg) or with corn oil for 7 days. The neurologic deficit score was assessed for neurological symptoms after global cerebral I/R. Levels of oxidative stress biomarkers were measured to evaluate the oxidative stress injury of hippocampal neurons. Western blot and Nissl staining were performed to assess the apoptosis of hippocampal neurons and test the expression of ERK-1/2 to investigate signal mechanism. Results: Treatment with 40 and 80 mg/kg allicin resulted in a significant decrease of deficit score in comparison to model group. 80 mg/kg allicin obviously elevated activities of SOD, reduced the level of MDA and attenuated apoptosis of hippocampal neurons in gerbils with I/R injury. In addition, 80 mg/kg allicin inhibited the I/R-induced reduction of ERK-1/2 phosphorylation level. Conclusion: Our results suggest that allicin alleviates I/R-mediated neuronal impairment by anti-oxidative and anti-apoptosis pathways, which is likely to be related to ERK-1/2 signal pathway.

Keywords: Allicin, Ischemia/reperfusion, oxidative stress, apoptosis, ERK-1/2

Introduction

Ischemic stroke is a leading cause of long-term disability and ranks second among all causes of death in adults worldwide [1]. Ischemic stroke results from a temporary or permanent reduction of cerebral blood flow that leads to functional and structural damage in different brain regions. Although reperfusion is critical for ischemic brain tissue to restore normal function, it can paradoxically result in secondary damage, called ischemia/reperfusion (I/R) injury [2]. The mechanisms leading to cellular damage from I/R injury are complex and multifactorial. Despite decades of intense research, the beneficial treatment of stroke remains limited [3]. Therefore, the search for effective means ameliorating cerebral I/R injury is one of the major problems of experimental medicine and biology [4].

Allicin, the main biologically active compound derived from garlic, exerts a broad spectrum of pharmacological activities such as anti-microbial [5, 6], anti-oxidative [7], anti-inflammatory [8, 9] and is considered to have therapeutic potential in many neurological disorders [10-12]. Considerable evidence indicates that oxidative stress plays an important role in the regulation of I/R injury [13, 14]. There are studies indicating that allicin decreases ROS generation and increases the level of glutathione through its antioxidative ability in endothelial cells [15]. However, to date, there are few reports about the mechanisms of allicin involved in I/R-induced cerebral impairment.

Table 1. The effects of allicin on neurological deficit scores in gerbils after global I/R

Group	Allicin dose (mg/kg)	Gerbil number (n)	Neurological deficit scores
Vehicle	0	6	0.00 ± 0.00
Model	0	6	2.83 ± 0.56*
Allicin	20	6	2.16 ± 0.67
	40	6	1.83 ± 0.16#
	80	6	1.17 ± 0.56##

Gerbils were treated with intragastric administration of allicin or corn oil after global I/R. Neurological deficit scores were assessed at 7th day after intragastric administration. *P < 0.05 vs. vehicle-treated group; #P < 0.05 vs. model group; ##P < 0.01 vs. model group.

Therefore, the present study was conducted to determine the effects of allicin on global cerebral I/R injury and clarify whether allicin could inhibit hippocampal injury-induced by global cerebral I/R by anti-oxidation and anti-apoptosis and related to ERK-1/2 signal pathway in gerbil.

Materials and methods

Subjects

Mongolian gerbil was used widely as a global brain ischemia/reperfusion model because of its incomplete cerebral circle of Willis. In this study, adult male Mongolian gerbils (60-80 g) were used and transient global ischemia was induced as described previously with minor modification [16]. Briefly, they were controlled in individual cages available for standard rat food and water. The holding room was kept at a constant temperature of 24°C, on 12:12 h light/dark cycle, 50-70% humidity. Gerbils were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). Transient global ischemia was induced by 7-min occlusion of bilateral common carotid arteries. Seven minutes later, cerebral blood flow was restored and the neck incision area was then sutured. The gerbils were kept under a heating pad and lamp for 2 h to prevent hypothermia. Vehicle-treated animals were treated identically, except that the bilateral common carotid arteries were not occluded. Efforts were made to minimize suffering and reduce the number of animals used. All experimental protocols were pre-approved by the Experimental Animal Ethic Committee of Harbin Medical University, China (Animal Experimental Ethical Inspection Protocol No. 2009104).

45 gerbils were randomly divided into five groups (n = 9): (1) vehicle-treated group, which was subjected to sham operation with intragastric (i.g.) administration of corn oil 1 ml/100 g/day; (2) model group, which underwent the occlusion of common carotid arteries and received vehicle i.g. (corn oil 1 ml/100 g/day); (3-5) allicin groups, which was subjected to the occlusion of common carotid arteries and intragastric treated with allicin 20, 40 and 80 mg/kg/day, respectively. Allicin (Sigma, with a purity > 90%) is dissolved in corn oil. All animals were treated with intragastric administration of allicin between 9:00 and 11:30 each morning.

Neurological function

At 7th day after intragastric administration, gerbils were assessed for neurological deficit, according to the following stroke index [17]: 0, no symptom; 1, hunched posture or hair roughed up; 2, ptosis; 3, circling behavior; 4, splayed-out hind limb; 5, seizures. The neurobehavioral scores of each gerbil of a group were added and averaged to obtain neurological deficit (ND) score of each group. The animals showing no sign of ND or < 3 were not included in the study. The 45 gerbils were sacrificed at the end of neurological deficit assessment. 3 of 9 gerbils in each group were used for Nissle staining. Because the pyramidal neurons of the CA1 region in the hippocampus are selectively vulnerable to transient global cerebral I/R damage [18], hippocampal tissues from another 6 gerbils in each group were for oxidative stress and western blot measurements.

Measurement of the indicators of oxidative stress in gerbils

The enzymatic activities of Cu/Zn-superoxide dismutase (SOD) and the level of malondialdehyde (MDA) were evaluated with different detection kits according to manufacturer's instructions (Nanjing Jian Cheng Bioengineering Institute). SOD activities were detected in hippocampus homogenate by measuring its ability to inhibit the nucleotide oxidation. Data were defined as SOD units/mg protein. MDA is a product of lipoperoxidation. The level of MDA in hippocampus was calculated by measuring thiobarbituric acid reacting substances at 532 nm with the use of U-2000 spectrophotometer. The concentration of MDA was expressed as nmol MDA per milligram protein.

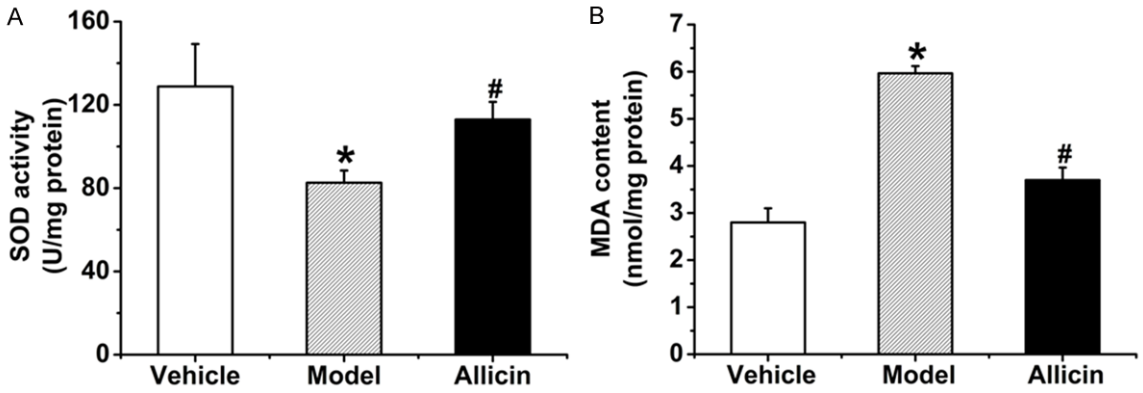

Figure 1. Effects of allicin on oxidative stress biomarkers, SOD (A) and MDA (B), in I/R gerbil hippocampus. *P < 0.05 compared to vehicle group; #P < 0.05 compared to model group.

Nissl staining

The animals in each group (n = 3) were anesthetized with 10% (v/v) chloral hydrate and transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4) for 10 min, followed by 4% paraformaldehyde in 0.1 M phosphate buffered (PB, pH 7.4) for fixation for 10 min. The brains were then removed, post-fixed in 4% paraformaldehyde for 48 h and then cryoprotected by infiltration with 30% sucrose for 3 days at 4°C. Coronal sections (8 μm) of the hippocampus were cut and submerged in 0.1% cresyl violet for 10 min at 37°C and then were rinsed in distilled water and dehydrated in graded ethanol covers lipped with neutral balsam.

Western blot analysis

Hippocampal tissues were homogenized with ice-cold lysis buffer (Tris 50 Mm, pH 7.4, NaCl 150 mM, Triton X-100 1%, glycerol 10%, Nonidet P-40 1%, EDTA 5 mM, and PMSF 2 mM). The lysates were centrifuged at 13,500 r.p.m for 15 min at 4°C, and the supernatant was collected and total protein content was determined using a BCA protein assay kit with bovine serum albumin as the standard (Beyotime Institute of Biotechnology, China). Samples with an equal amount of protein (50 μg) were separated in 10%-12% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes (Millipore, MA). The membranes were then blocked using 5% fat-free milk in Tris-buffered saline with 1% Tween-20 (TBS-T) for 1 h and then were incubated with the following primary antibodies: rabbit anti-caspase-3 (1:5000, Abcam), rabbit anti-Bcl-2 (1:200, Santa Cruz), rabbit anti-Bax (1:200, Santa Cruz), rabbit anti-phospho-ERK (1:1000, Cell Signaling), rabbit anti-ERK (1:1000, Cell Signaling), mouse anti-β-actin (1:2000, Kang Chen, China), respectively, overnight at 4°C. The membranes were washed with TBS-T, followed by the incubation with horseradish peroxidase-conjugated goat antirabbit antibody (1:5000, Santa Cruz) or goat antimouse antibody (1:5000, Santa Cruz), for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) kit (Pierce, CA) and exposed on an X-ray film. The immunoblots intensities were quantified using the Quantity One software (BioRad).

Statistical analysis

All data were analyzed using SPSS 15.0 software, and the results are expressed as the mean ± SEM. Statistical comparisons were performed with one-way analysis of variance (ANOVA), followed by Dunnett's or Student's t-test. P < 0.05 was considered to be statistically significant.

Results

Allicin improved neurological deficit scores in gerbils after global I/R

Allicin treatment (40 and 80 mg/kg/day, i.g) after I/R produced significant reduction in neurological score compared with the model group (n = 9, P < 0.05 or 0.01, **Table 1**), whereas allicin at 20 mg/kg/day did not cause significant reduction in neurological deficits. In order to acquire effective response, 80 mg/kg/day allicin was applied in all subsequent experiments.

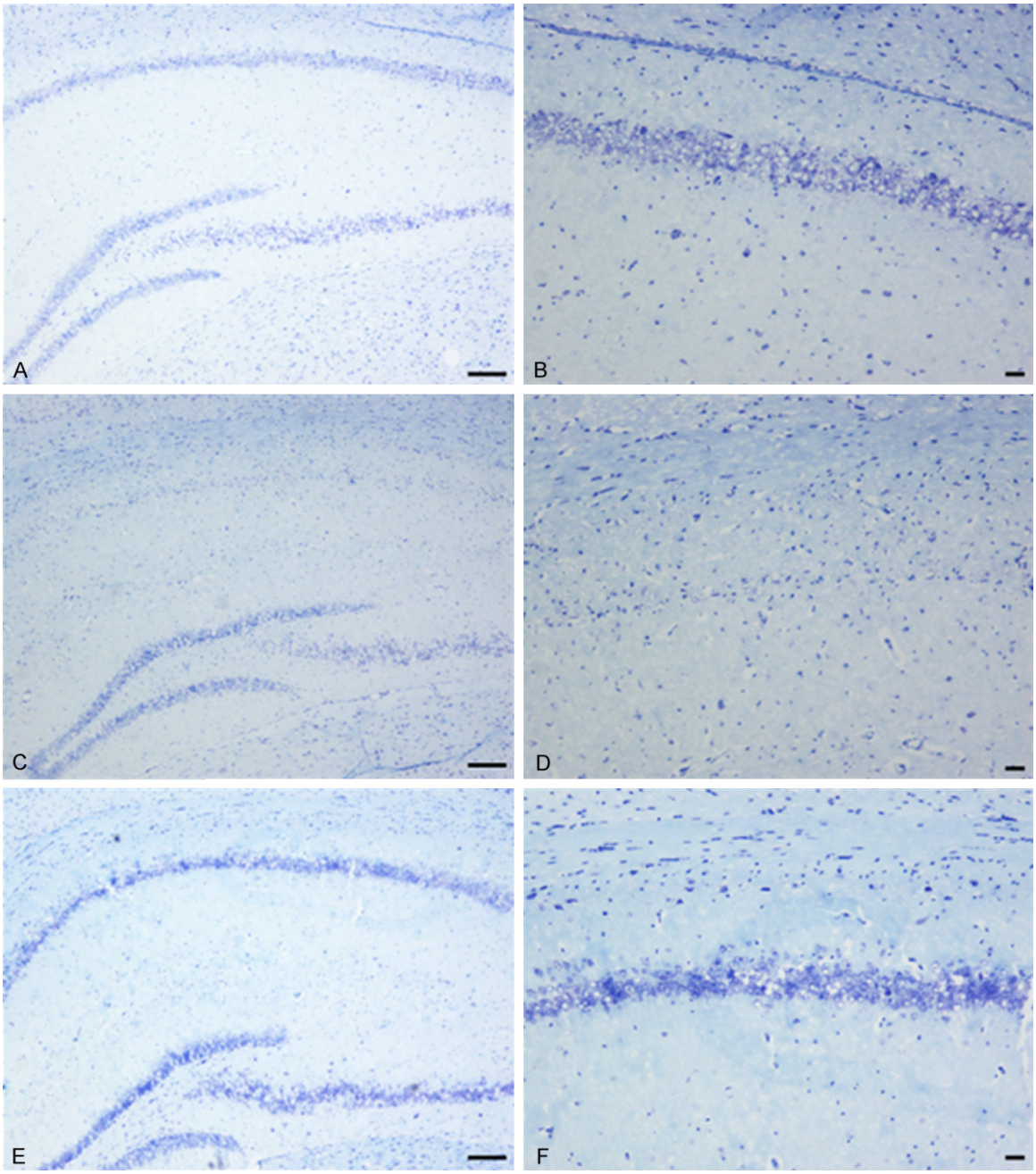

Figure 2. Nissl staining showed the neuroprotective effects of allicin on I/R-induced neuronal impairment of hippocampal CA1 region in gerbil. A, B. Neurons in vehicle group did not have obvious histopathological abnormalities. C, D. Neurons in model group exhibited significant cell damages with pycnotic nucleus or cell loss in CA1 region. E, F. Neuron survival with palely stained nuclei was markedly increased in 80 mg/kg/day allicin group compared with that in model group. Bar = 50 μm.

Allicin inhibited global I/R-induced oxidative stress in gerbil hippocampus

To explore whether allicin possessed neuroprotective effects via antioxidant mechanism, the oxidative stress biomarkers were detected in our study. **Figure 1A** showed that I/R markedly decreased the activities of SOD from 128.82 ± 20.40 to 82.58 ± 5.90 U/mg protein ($n = 6$, $P < 0.05$, compared with vehicle group). 80 mg/kg/day allicin treatment significantly increased SOD activity from 82.58 ± 5.90 to 112.96 ± 8.47 U/mg protein ($n = 6$, $P < 0.05$, compared with model group). Besides the concentration of MDA, an index of lipid peroxidation, was depicted. As shown in **Figure 1B**, the levels of

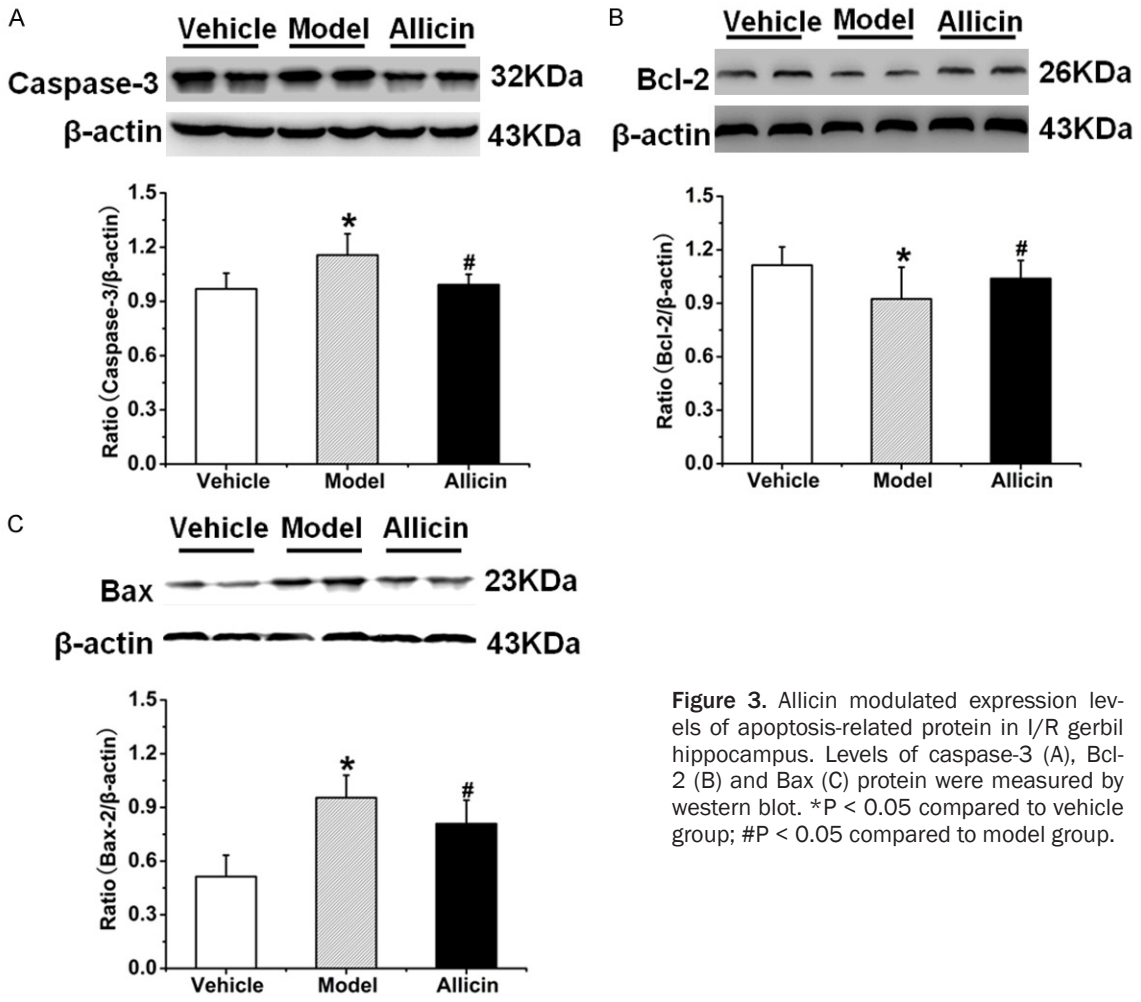

Figure 3. Allicin modulated expression levels of apoptosis-related protein in I/R gerbil hippocampus. Levels of caspase-3 (A), Bcl-2 (B) and Bax (C) protein were measured by western blot. *P < 0.05 compared to vehicle group; #P < 0.05 compared to model group.

MDA in model group was significantly increased from 2.80 ± 0.30 to 5.90 ± 0.15 nmol/mg protein (n = 6, P < 0.05, compared to vehicle group). 80 mg/kg/day allicin treatment significantly decreased MDA activity from 5.90 ± 0.15 to 3.70 ± 0.26 nmol/mg protein (n = 6, P < 0.05, compared with model group). These results suggest that allicin inhibits the I/R-induced oxidative stress in gerbil hippocampus.

Allicin protected hippocampal neuron against global I/R-induced apoptosis

The histological examination of Nissl staining was performed to evaluate I/R-induced a significant loss of gerbil's hippocampal CA1 region neurons. As shown in **Figure 2**, neurons in vehicle group did not have obvious histopathological abnormalities (**Figure 2A**, **2B**) and those in model group exhibited significant cell damages with pycnotic nucleus or cell loss in CA1 pyramidal neurons (**Figure 2C**, **2D**). However, in contrast to the model group, cell survival with palely stained nuclei was markedly increased by 80 mg/kg/day allicin treatment (**Figure 2E**, **2F**).

Furthermore, we evaluated whether allicin modulated the expression of apoptosis-regulatory indices, including caspase-3, Bcl-2 and Bax in gerbil hippocampus. The protein expression levels of caspase-3 (**Figure 3A**) and Bax (**Figure 3C**) were increased in model group in contrast to that in vehicle group (n = 6, P < 0.05). In 80 mg/kg/day allicin group, expression levels of the caspase-3 and Bax were significantly decreased in comparison with that in model group (n = 6, P < 0.05). As shown in **Figure 3B**, Bcl-2 protein level in model group was remarkably decreased compared with that in vehicle group (n = 6, P < 0.05). Treatment with 80 mg/kg/day allicin significantly elevated

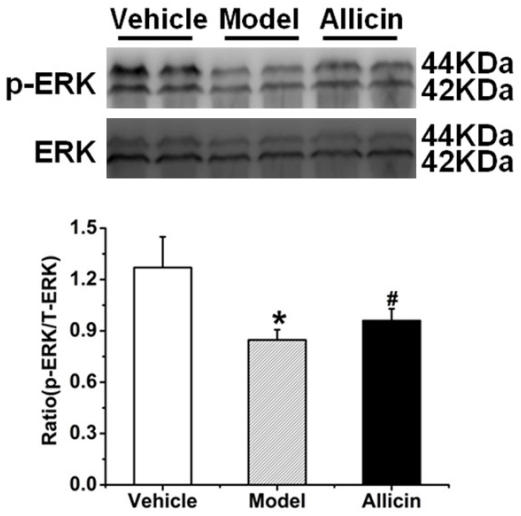

Figure 4. Allicin regulated phosphorylation levels of ERK-1/2 in I/R gerbil hippocampus. Western blot for phosphorylated and total ERK-1/2 in vehicle, model and allicin groups, respectively. *P < 0.05 compared to vehicle group; #P < 0.05 compared to model group.

the Bcl-2 protein expression in comparison with that in model group (n = 6, P < 0.05). Together, these data implied that allicin prevents gerbil hippocampus from apoptosis induced by global I/R.

Allicin influenced ERK-1/2 signaling pathway in global I/R gerbil hippocampus

To assess the possible involvement of ERK-1/2 activation in the effects of allicin on global I/R induced impairment, we examined ERK-1/2 signal activation in hippocampus by western blotting analysis. As shown in **Figure 4**, I/R reduced ERK-1/2 protein phosphorylation (p-ERK-1/2). p-ERK-1/2 was remarkably elevated in the gerbil treated with 80 mg/kg/day allicin in comparison with the model group (n = 6, P < 0.05). These results suggest that it is possible that ERK-1/2 is involved in the neuroprotective effects of allicin on global I/R-induced impairment.

Discussion

In this study, we observed that the effects of allicin on global cerbral I/R injury in gerbils. Mongolian gerbils were selected in the present study for inducing global ischemia because they possessed features of global cerebral ischemia just after brief occlusion of the carotid arteries due to the poor development of the circle of Willis [19, 20]. Allicin, the main biologically active compound derived from garlic, has been shown to exert various anti-oxidative and anti-apoptotic activities in in vitro and in vivo studies [11, 21-23]. But the effects and mechanisms of allicin on global cerebral I/R in gerbil have not been elucidated. We found allicin treatment improves significantly neurological function in I/R gerbils. Allicin treatment obviously elevated activities of SOD and reduced the level of MDA. Nissl staining results a marked suppression of neuronal cell death in the hippocampal CA1 area of I/R gerbils after treatment with allicin. The I/R-induced change of key apoptosis-related proteins, including caspase-3, Bcl-2 and Bax, were remarkably reversed in I/R gerbils treated with allicin. Allicin elevated the I/R-induced reduction of p-ERK-1/2 in gerbils.

Increasing evidence indicates that cerebral I/R causes oxidative damage in the central nervous system through increasing the level of reactive oxygen species (ROS) and lipid peroxidation [24]. Oxidative damage plays a crucial role in the neuronal injury caused by cerebral I/R and exerts its deleterious effects by oxidizing various cellular components. It can be estimated peroxidation of lipid bilayer which is a marker of oxidative stress by measuring the SOD and MDA levels [25]. MDA levels indirectly reflect the severity of attack in cells by free radicals and SOD activity levels indirectly reflect the capability of scavenging oxygen free radicals [26]. In present study, we found allicin enhanced the activities of SOD and decreased the concentration of MDA in I/R gerbils. These results implicated that neuroprotective effect of allicin on I/R induced neuron injury is related to its anti-oxidative effects.

Apoptosis, a form of programmed cell death, is directly or indirectly regulated tissue remodeling, aging and response, and irreversible damage. Abnormal apoptosis may be the cause of many diseases. Caspase-3 is considered the main terminal cleavage enzyme in the apoptosis process [27]. Both Bcl-2 and Bax are members of the Bcl-2 family that play pivotal roles in regulating mitochondria-mediated apoptotic pathway. Neuronal death or survival is determined by the balance between proapoptotic (Bax) and antiapoptotic (Bcl-2 and Bcl-xl) pro-

teins during cerebral ischemia [28]. Our results showed that allicin inhibited the enhancement of caspase-3 and Bax and the decrement of Bcl-2 induced by I/R in gerbil. These data indicated that allicin could prevent neurons from I/R injury by anti-apoptotic pathway.

Mitogen-activated protein kinases (MAPKs), including extracellular signal regulated kinase-1/2 (ERK-1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK, are mediators of various cellular signaling pathways in response to extracellular apoptotic stimuli and intracellular oxidative stress after transient focal cerebral ischemia [29, 30]. Considerable evidence showed that the MAPK, especially ERK-1/2 played a pivotal role in neurological function and neuronal impairment [31, 32]. However, the involvement of ERK-1/2 pathway in allicin-modulated neuroprotective effects on I/R in gerbil is still unclear. Our results showed that allicin inhibited the suppression of ERK-1/2 induced by global I/R in gerbil. It suggested that the neuroprotective effects of allicin on I/R may be related to ERK-1/2 signal pathway.

In summary, our results suggested that allicin alleviates global I/R-induced hippocampal neuronal impairment by anti-oxidative and anti-apoptotic effects and then improves neurological function, which is likely related to the ERK-1/2 signaling pathway in gerbil. This study further suggests the therapeutic potential of allicin in cerebral ischemic-reperfusion injury.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Li-Hui Qu, Department of Physiology, College of Basic Medical Sciences, Harbin Medical University-Daqing, 39 Xinyang Rd., Gaoxin District, Daqing, Heilongjiang 163319, P. R. China. Tel: +86 459 8153068, Fax: +86 459 8153366; E-mail: lihui_qu@aliyun.com

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