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Glycine confers neuroprotection through PTEN/AKT signal pathway in experimental intracerebral hemorrhage

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ABSTRACT

Glycine has been shown to protect against ischemic stroke through various mechanisms. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which antagonize Akt-dependent cell survival has been linked to neuronal damage. However, whether glycine has a neuroprotective property in intracerebral hemorrhage (ICH) was unknown. This study aimed to determine the protective effect of glycine in rats ICH. Adult male Sprague-Dawley (SD) rats were subjected to left striatum infusion of autologous blood. ICH animals received glycine (0.2–3 mg/kg, icv) at 1 h after ICH with or without pre-injection of Akt Inhibitor IV (100 μM, 2 μl, icv) 0.5 h prior to glycine treatment. Our results showed that in the perihematomal area PTEN was up-regulated in the early stage after ICH. However, glycine treatment decreased PTEN protein level and increased the phosphorylation level of AKT (p-AKT) in the peri-hematomal area. With the administration of glycine, neuronal death was significantly reduced and Evans blue leakage was alleviated as well as the brain edema after ICH. Moreover, hematoma volume was decreased and neurobehavioral outcome was improved. Nevertheless, Akt Inhibitor IV abolished the neuroprotective effects of glycine after ICH. Together, our findings demonstrate, for the first time, the protective role of glycine on ICH rats, and suggest that the neuroprotective effect of glycine was mediated through PTEN/Akt signal pathway.

1. Introduction

Intracerebral hemorrhage (ICH) is a stroke subtype that is associated with high mortality neurological impairments [1,2]. ICH accounts for 10–15% of all strokes in the USA, Europe and Australia and 20–30% of strokes in Asia, with about two million cases worldwide per year [3]. Despite its high fatality rate and disability rate, no efficient pharmacological preventions are available [4].

Glycine is the simplest non-essential amino acid which plays a fundamental role in cell metabolism. It is a major inhibitory neurotransmitter that binds to glycine receptor to inhibit post-synaptic neurons in the adult CNS. In the previous study, glycine has been shown to have neuroprotective effect in ischemia-reperfusion, anoxia, hypoxia and reactive oxygen species (ROS) [5–7]. A study of Gusev EI SV from Russia reports that glycine treatment shows significantly improved outcome and tended to decrease the 30 day mortality of ischemic stroke patients [8]. However, whether glycine has a neuroprotective property in hemorrhagic stroke is not known.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor with the important function that

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dephosphorylates both protein and lipid substrates [9,10]. PTEN regulates several basic cellular functions, such as cell cycle progression, cell migration, cell spreading and cell growth. It displays its lipid phosphatase activity by antagonizing Akt-dependent cell survival-promoting signaling via dephosphorylation of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) [9,11]. Akt is a multi-isoform serine/threonine kinase which plays a pivotal role in promoting cell survival and it is a direct downstream target of PI3K [10,12]. Our previous data indicate that PTEN inhibition by bpV(pis) enhancing Akt activation so as to confer neuroprotection in ischemia-reperfusion injury.

In addition to it, we also showed that glycine increases miR-301a expression which directly binds to PTEN 3′UTR target sites [13]. Glycine confers neuroprotection through microRNA-301a/PTEN signaling in rats cortical neurons in oxygen deprivation model [13]. Substantial studies indicate that inhibiting PTEN confers neuroprotection [10,14–16] in ischemia-reperfusion injury and traumatic brain injury [17,18]. Whether PTEN inhibition have protective role in ICH-induced brain injury were largely unknown.

In this study, we explored the protective role of glycine and PTEN inhibition on rats ICH.

2. Materials and methods

2.1. Animals and ICH model

All animal experiments carried out in compliance with the National Institutes of Health guidelines and the Animal Care and Ethics Committee of Wuhan University School of Medicine. Male rats (n = 256, weighing 280–300 g) in hemorrhagic stroke were induced using a modified double infusion model of autologous whole blood (80 μl) as previously reported [4]. Brain tissue of perihematomal regions were utilized for western blot and immunostaining analysis.

2.2. Glycine and Akt Inhibitor IV administration

Since glycine has limited ability to penetrate the blood-brain barrier, we administrated it with intracerebroventricular (icv) injection. Glycine (Sigma, USA) was dissolved in normal saline. After ICH-induction, animals were randomly treated with different dose of glycine at 1 h after surgery. Intracerebroventricular injection was performed as our previous study [19]. Akt Inhibitor IV (Santa Cruz, CA) were injected 0.5 h before glycine at the concentration of 100 μM (2 μl, icv) as reported in our previous study [20].

2.3. Western blotting

For the detection of p-Akt and AKT, the samples prepared in the same day were used. The PVDF membrane was incubated with primary antibody against phospho-Akt (Ser473) (1:1000, Cell Signaling Technology, Beverly, MA), Akt (1:1000, Cell Signaling Technology, Beverly, MA), PTEN(1:1000, Cell Signaling Technology, Beverly, MA), β-actin (1:2000, Santa Cruz Biotech). The quantification of Western blot data was performed using Image J software.

2.4. Immunofluorescece staining

Rats were sacrificed, perfusing with iced PBS followed by 4% PFA. Their brains were removed and postfixed with 4% PFA and sucrose. 3 brain slices were selected from the same part of one brain. Brain sections were incubated at 4 °C overnight with PTEN primary antibody (1:200, Cell Signaling Technology). The tissues were then incubated with a secondary antibody (Alexa-594 conjugated to goat anti-mouse) for 1 h. Then the sections were observed under an Olympus fluorescent microscope (IX51, Olympus) to observe the average number of positive cells.

2.5. Fluoro Jade-C (FJC) staining

FJC labeling was performed using a standard protocol with modification as it is reported before [21]. Three brain slices at the same site of each brain were randomly selected. Immersed brain slices in 1% sodium hydroxide in 80% ethanol for 5 min, followed by rinsing in 70% ethanol for 2 min, in distilled water for 2 min, and then incubated in 0.06% potassium permanganate solution for 10 min. After 2 min in distilled water, the slides were transferred into 0.0001% solution of FJC (Sigma Aldrich, USA) which was dissolved in 0.1% acetic acid. Slides were rinsed with distilled water for 1 min, three times, and then dried at 50 °C for 5 min. The data were expressed as FJC-positive neurons/(visual field). Four fields around the hematoma were selected for each brain slice to observe the average number of positive cells in the visual field.

2.6. Nissl staining

The brain samples were shared and similarly prepared as FJC staining. Nissl staining performed according to the manufacturer's instructions. Brain slices were selected from the same part in different brain tissues. The coronal brain sections were incubated with 100 μl nissl body staining solution (Wuhan Goodbio technology CO, LTD) for 5–10 min at room temperature. Then wash it with PBS 3 times. Treat the brain sections with 95% alcohol for 10–15 s, sealed with neutral gum and observed under an Olympus microscope. Three slides per animal were stained and images were obtained from 4 fields around hematoma per slide and the nissl body cells were counted as it was described in FJC staining.

2.7. Evans blue extravasation

Evans blue extravasation as reported previously [22]. Briefly, at the time of 24 h after ICH, the Evans blue dye (2%, 5 ml/kg; Aladdin, Shanghai, China) was injected and administered >2 min into the left femoral vein under anesthesia, where it was allowed to circulate for 60 min. Then brain samples were weighed, homogenized in sterile saline, and centrifuged at 15000 g for 30 min. Equal volume of trichloroacetic acid was added to the resultant supernatant. Those samples were then incubated overnight at 4 °C and centrifuged again at 15000 g for 30 min. The resultant supernatant was spectrophotometrically quantified for the Evans blue dye at 620 nm.

2.8. Brain water content

Brain water content was evaluated as previously reported [23]. All specimens were weighed as wet-weight, then dried in an oven at 100 °C for 72 h and weighed again as dry-weight. Brain water content was calculated as (wet weight − dry weight)/wet weight × 100%.

2.9. Hematoma volume analysis

Hematoma volume of ICH model was analyzed at 24 h after ICH as it was reported before [22]. After ICH rats were sacrificed, contiguous coronal slices of hematoma were prepared and the hematoma volume measured on images was calculated in a blinded manner by using Image J software (Image J, USA).

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