Original Article

Icariin protects against cerebral ischemia/reperfusion injury by activating the PI3K/Akt signaling pathway

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Abstract: Icariin has been proved to confer neuroprotection against cerebral ischemia/reperfusion (I/R) injury. However, the exact mechanisms remain unclear. We therefore investigated the neuroprotective role of icariin and the underlying mechanisms in the current study. Focal transient cerebral I/R model was induced by 2 h of ischemia followed by 24 h reperfusion in mice. The animals were pretreated with ICA at a dose of 30 mg/kg once per day for 5 consecutive days followed by cerebral I/R injury. Neurological function, infarct volume, brain edema and apoptosis were measured at 24 h after reperfusion. Furthermore, PI3K inhibitor LY294002 was injected into the lateral cerebral ventricle 15 min before ischemia to evaluate the underlying mechanisms. Compared with the vehicle-treated group, icariin treatment significantly ameliorated neurological deficit, infarct volume, and brain edema, and reduced neuronal apoptosis. However, those protective effects of icariin were abolished by LY294002 (LY). Additionally, icariin up-regulated p-Akt expression, which was inhibited by LY. In conclusion, our results suggest that icariin protects the brain against I/R injury via the activation of PI3K/Akt signaling pathway.

Keywords: Cerebral ischemia, icariin, neuroprotection, PI3K/Akt signaling

Introduction

Ischemic stroke is a severe health problem with high mortality and morbidity and it remains the third leading cause of death all around the world [1]. The treatment for ischemic stroke is thrombolysis as soon as possible, however, reperfusion can bring about ischemia/reperfusion (I/R) injury, which causes more neuronal death. The mechanisms of I/R injury include oxidative stress, excitotoxicity, inflammation, and apoptosis [2]. Despite considerable progress has been made to alleviate injury in cerebral I/R injury, the effective therapeutic strategies are still limited, indicating that novel targets and drugs are urgently needed.

Icariin, a flavonoid derived from Herba Epimedii, is the main active ingredient of a traditional Chinese medicine called Yin Yang Huo. Icariin has been suggested to confer various pharmacological effects, including anti-inflammation, anti-oxidation, anti-apoptosis, and anti-cancer [3-5]. It has been reported that icariin exerts protection against cerebral I/R injury [6, 7]. Additionally, PI3K/Akt signaling activation has been indicated to be protective against cerebral I/R injury [8]. However, whether PI3K/Akt signaling is activated in the neuroprotective effects of icariin remains unclear. Therefore, the present study aims to explore whether icariin protects against cerebral I/R injury by activating the PI3K/Akt signaling pathway.

Materials and methods

Animals

Male C57BL/6 mice (25-30 g) were purchased from the Experimental Animal Center of the Xi’an Jiaotong Medical University. All experiment procedures were according with the National Institutes of Health (NIH) Guidelines on the Use of Laboratory Animals, and were approved by the Xi’an Jiaotong Medical University Committee on Animal Care. All animals were allowed free access to food and water in a temperature-controlled room (22±3°C) with a 12-h light-dark cycle.
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**Experimental groups and protocols**

Mice were randomly divided into four different groups: (1) Sham group; (2) vehicle-treated cerebral ischemia/reperfusion (I/R) group (I/R + vehicle group); (3) icariin-treated I/R group (I/R + icariin group); (4) I/R + icariin + LY294002 (LY) group. Mice from (3) and (4) groups were administered with icariin at a dose of 20 mg/kg by gavage once per day for 5 consecutive days, while mice from (1) and (2) groups were administered equal volume of normal saline. LY (PI3K/Akt inhibitor, 10 mmol/L) was dissolved in 2% DMSO. LY of 5 μl was injected into the lateral cerebral ventricle 15 min before ischemia.

**Animal model of transient focal cerebral ischemia**

Middle cerebral artery occlusion (MCAO) was performed as previously reported [9]. Briefly, mice were anesthetized with sodium pentobarbital (40 mg/kg i.p.). A 6-0 rounded tip nylon monofilament was gently advanced from the right common artery to the internal carotid artery. Focal cerebral ischemia for 2 h was induced by MCAO and reperfusion for 24 h was induced by withdrawing the suture. The same surgical procedures were performed on sham animals without MCAO. The mice were allowed to recover in a cage with free access to food and water in a temperature-controlled and air-ventilated room.

**Neurological score assessment**

Neurological deficits were evaluated by an observer blinded to the treatment of animals after 24 h reperfusion as previously described [10]. 0, no neurologic deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, falling to the left; 4, did not walk spontaneously and had a depressed level of consciousness.

**Evaluation of cerebral infarct volume**

The animals were euthanized 24 h after reperfusion. The brains were rapidly dissected out and the forebrains were cut into six coronal sections (2 mm thick). The sections were stained by incubating them in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min. The cross-sectional areas with or without infarction in each brain slice were measured using Image J analysis software (version 1.6 NIH).

**Evaluation of brain edema**

As described previously, after the wet weight of the brain tissues was quantified, the red and white parts of these brains were desiccated at 105°C for 48 h until the weight was constant to obtain the dry weight [11]. The water content of each brain was calculated as follows: (wet weight-dried weight)/wet weight ×100%.

**Nissl staining and TUNEL staining**

Neuronal death was assessed by Nissl staining. At 24 h after reperfusion, mice were sacrificed and perfused with 4% paraformaldehyde. Brains were post-fixed in the same fixative for 1 day and then embedded in paraffin. Then, the brains were sectioned at a 2-μm thickness. Nissl staining was performed according to the manufacturer’s protocol (Beyotime). The brain sections were observed under a light microscope. Neuronal apoptosis was assessed by TUNEL staining. The brain sections were stained by using TUNEL apoptosis assay kit (Beyotime) and observed under a light microscope. Apoptotic index was determined as the ratio of the number of TUNEL-positive neurons to the total number of neurons.

**Western blot**

Total protein extraction was performed at 24 h after reperfusion according to the manufactur-
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The protein concentrations were determined using a BCA Protein Assay reagent kit (Beyotime). Equal amounts of protein were separated by SDS/PAGE and transferred on to PVDF membranes. After being blocked with 5% nonfat dry milk in TBST for 2 h, membranes were incubated with primary antibodies against Akt and phospho-Akt at overnight 4°C. Then, the membranes were washed with TBST and incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h at room temperature. The protein bands were detected using a chemiluminescence system, and the bands were analyzed using Quantity One System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data is presented as means ± S.D. and analyzed by SPSS 19.0 software (SPSS, USA). The significance of differences among groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s t-test for intergroup comparisons. For all test, P < 0.05 was considered as statistically significant.

Results

Effects of icariin on neurological deficits

No neurological deficits were detected in the sham group. At 24 h after reperfusion, I/R mice showed severe neurological deficits. Icariin treatment significantly improved the neurological score compared with the I/R + vehicle group (P < 0.05). However, LY treatment abolished the protective effects of icariin compared with the I/R + icariin group (Figure 1).

Effects of icariin on cerebral infarct volume

No infarct area was observed in the sham group, while in the I/R group, a dramatic infarction was observed at 24 h after reperfusion. Icariin treatment significantly decreased the infarct volume in the I/R + icariin group compared with that in the I/R + vehicle group (P < 0.05). However, LY treatment increased the infarct volume markedly in comparison to the I/R + icariin group (Figure 2).
Effects of icariin on brain edema

At 24 hours after reperfusion, the brain water content in the ischemic area of the I/R + vehicle group was significantly higher than that of the sham group. Pretreatment with icariin significantly reduced brain water content compared with that in the I/R + vehicle group ($P < 0.05$), which was abolished by LY treatment ($P < 0.05$) (Figure 3).

Effects of icariin on neuronal death

As shown in Figure 4, intact neurons were observed in the sham group, while many damaged neurons with condensed cytoplasm and irregular cell arrangements in I/R + vehicle group were detected ($P < 0.05$). The neuronal death was alleviated by icariin treatment ($P < 0.05$). However, this protective effect was abolished by LY treatment ($P < 0.05$) (Figure 4).

Effects of icariin on neuronal apoptosis

As shown in Figure 5, I/R + vehicle group exhibited many stained, condensed nuclei, indicating the apoptotic neurons. The number of apoptotic neurons decreased in I/R + icariin group compared with the I/R + vehicle group ($P < 0.05$). On the contrary, LY treatment increased...
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Effects of icariin on p-Akt expression

The results of Western blot suggested that I/R injury markedly increased Akt phosphorylation relative to the sham group. Akt phosphorylation was further enhanced with icariin treatment. However, LY abolished the increase in p-Akt when co-administered with icariin (Figure 6).

Discussion

In the current study, we find that icariin attenuates brain injury induced by cerebral I/R injury. Icariin ameliorates neurological deficits, decreases infarct volume, brain water content, and neuronal death. However, these protective effects of icariin were abolished by treatment with LY, a PI3K signaling inhibitor, indicating that icariin confers neuroprotection via the activation of PI3K/Akt signaling.

Ischemic stroke causes severe brain injury in millions of people all over the world every year. Although many advances have been made in the pathogenesis of stroke, the effective therapy is urgently needed to be developed. The use of rt-PA has been used in the treatment of stroke, but the prognosis is limited due to various reasons. Cerebral I/R injury is one of the important factors, which can lead to the aggravation of brain injury. The mechanisms of cerebral I/R injury include inflammation, oxidative and nitrative stress, and neuronal death. Glutamate has been suggested to induce neuronal death in ischemic stroke [12]. Exposure of neurons to excitotoxic levels of glutamate results in an initial transient increase in cytosolic calcium concentration followed by a delayed sustained rise in cytosolic calcium concentration, which is believed to induce neuronal death [13]. Additionally, apoptosis is a major form of neuronal death in stroke. The two pathways of apoptosis include mitochondrial and death-receptor signal pathways [14]. Mitochondrial pathway is initiated by the release of cytochrome C from the mitochondria. Then, cytochrome C activates caspase cascade, causing apoptosis [15]. The results of the present study suggest that icariin decreases the number of TUNEL-positive neurons, indicating that icariin inhibits neuronal apoptosis against cerebral I/R injury.

The formation of cerebral edema in ischemic stroke is associated with the disruption of blood-brain barrier (BBB). The breakdown of BBB can lead to severe neurologic deficits via aggravation of edema formation and brain hemorrhage [16, 17]. The integrity of BBB is maintained by various junction proteins, such as ZO-1, occludin, and VE-cadherin. Ischemic stroke induces occludin degradation, resulting in BBB damage [18]. BBB damage then aggravates brain edema and injury. In the present study, we found that icariin treatment decreases brain water content, suggesting that icariin ameliorates BBB leakage.

Icariin has been reported to exhibit a wide range of pharmacological effects. Pan et al. suggested that icariin possessed an antidepressant-like property [19]. Nian et al. reported that icariin has a definite antiosteoporotic effect, similar to estrogen [20]. Yang et al. suggested that icariin promotes cell proliferation in human neural stem cells [21]. Wei et al. found that icariin prevents cartilage and bone degradation in experimental models of arthritis [22]. Furthermore, icariin is suggested to protect against brain injury by enhancing SIRT1-dependent PGC-1alpha expression in experi-

Figure 6. Effect of icariin on the activation of Akt following cerebral I/R injury. Representative images of the Western blot results were shown. Data were expressed as mean ± S.D. (n = 6 in each group); *P < 0.05 versus the sham group, #P < 0.05 versus the I/R + vehicle group, $P < 0.05 versus the I/R + icariin group.
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mental stroke [7]. The results of the current study show that the protective effect of icariin is abolished by LY, suggesting that icariin protect against brain injury by activating PI3K/Akt signaling pathway.

PI3K/Akt signaling, a well-conserved family of signaling transduction pathway, plays a critical role in regulating cell proliferation and survival [23, 24]. The PI3Ks and the downstream serine/threonine kinase Akt can regulate inflammatory responses, and apoptosis [23]. More importantly, PI3K/Akt signaling has been reported to confer protection against cerebral I/R injury. PI3K/Akt pathway has been suggested to be involved in the neuroprotective effect of Tongxinluo against focal cerebral I/R injury [25]. H2S inhalation protects against cerebral I/R injury through PI3K/Akt/Nrf2 pathway [26]. The current study confirmed that icariin activates PI3K/Akt signaling, further increases the phosphorylation of Akt. The inhibitor of PI3K attenuates the protection of icariin, indicating that icariin exerted protective effects via PI3K/Akt signaling.

In summary, our study suggests that icariin protects against cerebral I/R injury via PI3K/Akt signaling pathway. Icariin has a significant clinic significance against stroke and should be developed into a neuroprotectant for ischemic brain injury.

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Disclosure of conflict of interest

None.

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