Aescin Protects Neuron from Ischemia-Reperfusion Injury via Regulating the PRAS40/mTOR Signaling Pathway

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Ischemic stroke is one of the major causes of disability; widely use of endovascular thrombectomy or intravenous thrombolysis leads to more attention on ischemia-reperfusion injury (I/R injury). Aescin, a natural compound isolated from the seed of the horse chestnut, has been demonstrated anti-inflammatory and antiedematous effects previously. This study was aimed at determining whether aescin could induce protective effects against ischemia-reperfusion injury and exploring the underlying mechanisms in vitro. Primary cultured neurons were subjected to 2 hours of oxygen-glucose deprivation (OGD) followed by 24 hours of simulated reperfusion. Aescin, which worked in a dose-dependent manner, could significantly attenuate neuronal death and reduce lactate dehydrogenase (LDH) release after OGD and simulated reperfusion. Aescin treatment at a concentration of 50 μg/ml provided protection with fewer side effects. Results showed that aescin upregulated the phosphorylation level of PRAS40 and proteins in the mTOR signaling pathway, including S6K and 4E-BP1. However, PRAS40 knockdown or rapamycin treatment was able to undermine and even abolish the protective effects of aescin; meanwhile, the levels of phosphorylation PRAS40 and proteins in the mTOR signaling pathway were obviously decreased. Hence, our study demonstrated that aescin provided neuronal protective effects against I/R injury through the PRAS40/mTOR signaling pathway in vitro. These results might contribute to the potential clinical application of aescin and provide a therapeutic target on subsequent cerebral I/R injury.

1. Introduction

Stroke is a major cause of disability globally; 85% are ischemic and responsible for approximately 5 million deaths every year [1, 2]. The commonest cause of ischemic stroke is pathophysiological thrombosis or thromboembolism giving rise to cerebral arterial occlusion [3]. Nowadays, endovascular thrombectomy and intravenous thrombolysis have been widely recommended and performed to treat patients with acute ischemic stroke. However, not all patients end up with good outcomes after endovascular thrombectomy or intravenous thrombolysis. Apart from some known complications, such as intraoperative hemorrhage, subsequent ischemia-reperfusion injury (I/R injury) may be one of the most important factors contributing to the poor prognosis [4]. Owing to that I/R injury is a series of complex pathophysiological mechanisms and involves various signaling pathways [5–8], till now, no effective measures have been developed for cerebral I/R injury in a clinic.

Aescin is a natural compound isolated from the seed of the horse chestnut [9]. Studies have demonstrated the anti-inflammatory, antiedematous, and neuroprotective effects of aescin. It is reported that aescin treatments could provide a neuroprotective effect via inhibiting cell apoptosis [10]. Also, it is capable to inhibit the increase of capillary permeability, attenuate cerebral edema, and reduce the adhesiveness and
migration of neutrophils induced by various factors [11–13].
To date, the majority of researches about aescin stated the application on cerebral trauma and intracranial hemorrhage.
Nevertheless, limited comprehensive studies have been carried out in vitro or in vivo to address whether it can be applied to administrate subsequent cerebral I/R injury.

Accumulating evidences suggest that the neurobiological function of aescin is related to the Akt and mammalian target of rapamycin (mTOR) signaling pathway [14, 15]. For instance, aescin protected retinal pigment epithelium cells from oxidative stress via activating the Akt signaling pathway [16]. In Huntington’s disease, it promoted neurodegeneration through mTOR signaling pathways [15]. Our previous studies found that the mTOR signaling pathway played an important role in the protection against brain I/R injury both in vitro and in vivo [17, 18]. Additionally, the proline-rich Akt substrate of 40 kDa (PRAS40) played a pivotal role in protecting against I/R injury by linking the Akt and mTOR pathways [19]. According to all of these findings, we supposed that there was a potential association between aescin and the PRAS40/mTOR pathway.

In this study, we used the oxygen-glucose deprivation (OGD) and reperfusion model in primary cultured neurons to simulate the neuronal I/R injury in vitro and examined whether aescin treatment could alleviate neuronal I/R injury. What is more, we also try to figure out whether the neuroprotective effects of aescin are mediated by regulating the PRAS40/mTOR signaling pathway.

2. Materials and Methods

2.1. Animals. All pregnant C57BL/6 mice were purchased from Slac Laboratory Animal Co. (Shanghai, China) and maintained in the animal facility at Fudan University. All experimental protocols were approved by the Fudan University Department of Laboratory Animal Science. Mice were allowed free access to food and water before all procedures.

2.2. Primary Neuronal Cultures. The methods for the culture of well-characterized embryonic mice primary cortical neurons are described in detail in published works [20]. Timed-pregnant C57BL/6 mice (E16-17) were euthanized with isoﬂurane, and the embryos were removed from the uterus. The scalp and skull were cut. The cerebral cortex was freshly dissected and placed in a dish containing cold DMEM/F12 (Gibco) culture medium without calcium and magnesium. Meninges and blood vessels were carefully peeled off under a microscope. Then, the cortices were cut into pieces, digested in papain for 10 min at 37°C, and shaken two to three times by Pasteur pipettes. Complete media containing 10% fetal bovine serum were added to terminate digestion. Tissues were pipetted about 10 times slightly using the sterile Pasteur pipettes. Cell suspension was allowed to stand for 2 min, collected in a new 50 ml centrifuge tube, and filtered with a 40 μm cell strainer. Repeat the above step twice. Then, cell suspension was centrifuged at 1000 rpm/min at 4°C for 5 min. The supernatant was discarded. Cells were resuspended in the DMEM/F12 culture medium containing 10% fetal bovine serum. The cell resus-
was incubated with the reaction mixture from the kit for 30 min. LDH activity was determined via a colorimetric assay (Bio-Rad Laboratories, Hercules, CA). After the removal of the incubation buffer from a 96-well plate, 1% Triton X-100 lysing solution was applied to the remaining cells. The percentage of LDH released to the incubation buffer compared to total LDH was calculated as follows:

\[
\text{LDH release ratio} = \frac{\text{released LDH in buffer}}{\text{released LDH in buffer} + \text{intracellular LDH}}
\]

(1)

2.7. Protein Preparation and Western Blotting. To investigate the effects of aescin on the protein expression of the PRAS40/mTOR signaling pathway after OGD and reperfusion, whole-cell protein was extracted and western blot was performed as described before [22]. Briefly, primary cultured neurons grown in 6-well plates were harvested 24 hours after OGD and reperfusion and homogenized in cold RIPA buffer (9806; CST, Danvers, MA, USA), containing 1 mmol/l PMSF and a protease inhibitor cocktail (1:20, Cat# P-2714; Sigma, St. Louis, MO, USA). Simultaneously, the protein of the control group was also prepared for western blot. The homogenate was centrifuged at 12000 rpm for 15 min at 4°C, and the supernatant was collected for protein detection. 25 μg of protein was loaded into each lane and subjected to SDS-PAGE using 4%-15% Ready Gel (Catalog #L050505A2; Bio-Rad, Hercules, CA, USA) at 120 V for 120 min. Protein bands were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) at 250 mA for 3.5 hours. Membranes were incubated overnight with primary antibodies at 4°C followed by HRP-labeled secondary antibody (Invitrogen, Eugene, OR, USA) for 1 hour at room temperature. All the primary antibodies used are listed in Table 1. Membranes were scanned using Typhoon Trio (GE Healthcare, Amersham, Buckinghamshire, UK). Optical densities of all protein bands were analyzed using the IMAGEQUANT 5.2 software (GE Healthcare).

2.8. Statistical Analyses. The GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Student’s t-tests were used when 2 groups were compared. One-way or two-way Analysis of Variance (ANOVA) was used for multiple comparisons followed by the post hoc test. Differences were considered statistically significant at P values < 0.05. All data are expressed as mean ± SEM.

3. Results

3.1. Aescin Provided Protective Effects of Neuron against I/R Injury In Vitro. To determine whether aescin treatment can provide protective effects against I/R injury, we applied the OGD and reperfusion model to simulate the situation of neuronal I/R injury in vitro. Moreover, we treated primary cultured neurons with different concentrations of aescin after OGD and reperfusion to identify the appropriate dose. As displayed in Figure 1(a), primary neurons treated with aescin showed a less injury than the OGD group. Treatment with 25 μg/ml, 50 μg/ml, and 100 μg/ml aescin significantly reduced the OGD and reperfusion-induced LDH release compared to the control group, while the concentration of 5 μg/ml and 10 μg/ml did not (Figure 1(b)). We supposed that aescin worked in a dose-dependent manner. Furthermore, at a concentration of 50 μg/ml, aescin treatment decreased the LDH-releasing ratio with less cytotoxicity after OGD and reperfusion (n = 6). Thus, we selected the dose of 50 μg/ml aescin for subsequent experiments.

3.2. Aescin Treatment Increased the Phosphorylation of the PRAS40/mTOR Signaling Pathway after OGD and Reperfusion. We examined the expression of phosphorylated and nonphosphorylated PRAS40, mTOR, and the downstream proteins to confirm whether the PRAS40/mTOR pathway was involved in the protective effects of aescin. The western blot results showed that the phosphorylation level of PRAS40 and mTOR was downregulated after OGD and reperfusion, as well as S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1), the downstream of mTOR signaling pathway (Figure 2(a)). Aescin treatment could partly reverse this situation and significantly promote the phosphorylation of the PRAS40, mTOR, S6K, and 4E-BP1 compared with the OGD group (Figure 2(b)) (n = 6). However, aescin treatment does not alter the total expression level of these proteins. These results suggest that the aescin treatment may protect neurons against OGD and reperfusion-induced injury by increasing the phosphorylation of the PRAS40/mTOR signaling pathway.

3.3. PRAS40 Overexpression Protected Neuron against OGD and Reperfusion Injury and Promoted the Phosphorylation of the mTOR Signaling Pathway. To determine the role of PRAS40 in I/R injury, lentivirus overexpressing PRAS40 was transferred to primary cultured neurons. The injury degree of a neuron was evaluated by the LDH-releasing ratio as before. Compared with the control vector group, PRAS40 gene transfer reduced the LDH-releasing ratio (n = 6, P < 0.05), which indicated that PRAS40 overexpression could ameliorate the injury degree of neurons (Figure 3(a)). The phosphorylation of the mTOR signaling pathway was also examined by western blot. After OGD and reperfusion, PRAS40 overexpression could increase the level of phosphorylated mTOR, S6K, and 4E-BP1 (Figures 3(b) and 3(c)), while the phosphorylation of them was downregulated in the control vector group. The results indicated that PRAS40 overexpression may improve the survival rate of neurons via increasing the phosphorylation of the mTOR signaling pathway.

3.4. PRAS40 Knockdown Resulted in Severe I/R Injury and Less Phosphorylation of the mTOR Signaling Pathway. After demonstrating the effects of PRAS40 overexpression, lentivirus containing PRAS40 shRNA was used to consolidate the effects of PRAS40 knockdown after OGD and reperfusion. The LDH-releasing ratio significantly increased in the PRAS40 knockdown group than the control vector group after OGD and reperfusion (n = 6, P < 0.05) (Figure 4(a)). These results indicated that PRAS40...
Table 1: Antibodies, concentrations, and manufacturers used.

| Antibodies                  | Source | Dilutions | Manufacturer | Catalog# |
|-----------------------------|--------|-----------|--------------|----------|
| Phospho-mTOR (Ser2448)     | Rabbit | 1 : 1000  | CST          | 2971     |
| mTOR                       | Rabbit | 1 : 1000  | CST          | 2983     |
| Phospho-S6K p70 (Ser371)   | Rabbit | 1 : 1000  | CST          | 9208     |
| S6K p70                    | Rabbit | 1 : 1000  | CST          | 9202     |
| Phospho-PRAS40 (Thr246)    | Rabbit | 1 : 1000  | CST          | 13175    |
| PRAS40                     | Rabbit | 1 : 1000  | CST          | 2691     |
| Phospho-4E-BP1 (Thr37/46)  | Rabbit | 1 : 1000  | CST          | 2855     |
| 4E-BP1                     | Rabbit | 1 : 1000  | CST          | 9644     |
| β-Actin                    | Mouse  | 1 : 5000  | Sigma        | A-5441   |

![Figure 1: Neuroprotective effects of aescin treatment in vivo. (a) Primary neurons matured after 7 days from isolation. Neurons were subjected to 2 hours of OGD followed by 24 hours of reperfusion to simulate I/R injury in vitro. Neurons treated with aescin showed less axonotmesis compared with the OGD group. (b) LDH release was measured 24 hours after the experiments. All data after OGD and reperfusion were normalized to the values of the sham group (no OGD). After 2 hours of OGD and 24 hours of reperfusion, the LDH release ratio was significantly increased compared to the sham group. Aescin was administrated at various concentrations (5-100 μg/ml); treatments at concentrations of 25 μg/ml, 50 μg/ml, and 100 μg/ml aescin had protective effects in the form of reducing the LDH release induced by OGD and reperfusion compared with the no aescin treatment group (0 μg/ml). Neither 5 μg/ml nor 10 μg/ml aescin administration showed obviously protective effects, which means it worked in a dose-dependent manner. Furthermore, aescin at a concentration of 50 μg/ml resulted in a greater protective effect with less cytotoxicity after OGD and reperfusion. Values are mean ± SE. *P < 0.05 vs. sham (no OGD); †P < 0.05 vs. no aescin treatment (0 μg/ml). n = 6 per group.](image)
Figure 2: Aescin treatments upregulated the phosphorylation of PRAS40, mTOR, S6K, and 4E-BP1 in neurons after OGD and reperfusion. (a) Representative protein bands of critical molecules in the PRAS40/mTOR pathways were measured by western blot. The results showed the protein bands of phosphorylated and nonphosphorylated PRAS40, mTOR, S6K, and 4E-BP1 after 2 hours of OGD followed by 24 hours of reperfusion with 50 μg/ml aescin treatment or without aescin treatment in primary cultured neurons. Protein bands of the sham group (no OGD) were also displayed. β-Actin was used as a control to ensure equal protein loading. (b) The bar graphs represented the relative quantified protein levels of the phosphorylated and nonphosphorylated PRAS40, mTOR, S6K, and 4E-BP1, respectively. All results are given as means ± SEM. *P < 0.05 vs. sham (no OGD); †P < 0.05 vs. aescin treatment (50 μg/ml). n = 6 per group. Sham group: no OGD samples; Con group: OGD/R samples without treatment; NS group: OGD/R samples treated with normal saline; aescin group: OGD/R samples treated with aescin.
knockdown could aggravate the injury of neurons after the OGD and reperfusion. Nonphosphorylated and phosphorylated proteins in the mTOR signaling pathway were measured in the same method. As shown in Figures 4(b) and 4(c), the phosphorylation of mTOR, S6K, and 4E-BP1 in the PRAS40 knockdown group was significantly lower than that in the control vector group after OGD and reperfusion. This may be the underlying mechanism of an increased neuronal death rate.

3.5. PRAS40/mTOR Signaling Pathway Is Essential for the Protective Effects of Aescin against I/R Injury. Studies mentioned above have suggested that aescin could reduce the OGD and reperfusion-induced injury in primary cultured neuron cells and activate PRAS40 through phosphorylation. To further confirm the mechanism of aescin, we applied aescin accompanied with PRAS40 shRNA to examine whether the alteration of PRAS40 expression could undermine the neuroprotective effects of aescin. According to our results,
Aescin treatment decreased LDH release after OGD and reperfusion. In contrast, PRAS40 knockdown significantly increased the level of LDH release even with aescin treatment, which means it abolished the protective effects of aescin (Figure 5(a)) \((n = 6)\). Moreover, the phosphorylation of proteins in the mTOR signaling pathway was inhibited because of PRAS40 knockdown (Figures 5(b) and 5(c)).

Rapamycin was given to testify whether the protective effects of aescin were correlated to the mTOR signaling pathway. The group treated simultaneously with aescin and rapamycin showed a higher LDH-releasing ratio compared with the aescin treatment group (Figure 5(a)). Western blot data showed that rapamycin could obviously block the mTOR signaling pathway, which led to a stable lower phosphorylation level of proteins in the mTOR signaling pathway (Figures 5(d) and 5(e)). These results suggested that the protective effects of aescin treatment were closely associated with the activation of the PRAS40/mTOR signaling pathway.

4. Discussion

Stroke has become the second leading cause of death and the most common cause of complex chronic disability worldwide [2]. In a clinic, widely use of endovascular thrombectomy
Figure 5: Continued.
and intravenous thrombolysis leads to more attention on cerebral I/R injury until now. In order to testify our hypothesis, we chose the OGD and reperfusion model in primary cultured neurons to simulate artery recanalization-induced cerebral I/R injury in vitro. As our results showed, aescin treatment obviously decreased the LDH-releasing ratio after 2 hours of OGD followed by 24 hours of reperfusion, which indicated that aescin was capable to protect neurons against OGD and reperfusion-induced injury. Meanwhile, our findings demonstrated that aescin worked in a dose-dependent way. At a dose of 50 μg/ml in vitro, aescin could provide neuroprotective effects with less side effects. Beyond this concentration, the more aescin was administrated, the more cytotoxicity was presented.

PRAS40, a proline-rich Akt substrate, was identified as a regulator of the mTOR signaling pathway [29]. PRAS40 in conjunction with mTOR is closely associated with diabetes, cardiovascular diseases, and neurological diseases, such as improving insulin sensitivity [30], decreasing cardiomyocyte apoptosis after myocardial infarction [31] and neuroprotection after transient focal cerebral ischemia [32]. Our study suggested that aescin could significantly upregulated the phosphorylation level of PRAS40 and mTOR in primary cultured neurons after OGD and reperfusion, while the phosphorylated PRAS40 and mTOR was decreased without aescin treatment. S6K and 4E-BP1 are considered as the downstream effectors of the mTOR signaling pathway [33]. After OGD and reperfusion; the phosphorylated S6K and 4E-BP1 had the same trend with phosphorylated PRAS40 and mTOR; both of them were upregulated after aescin treatment compared with the OGD and reperfusion group.

However, the role of PRAS40 in the mTOR signaling pathway is still controversial [34]. Some of the studies stated...
that mTOR activity in some cell lines was inhibited by PRAS40 overexpression [35]: silencing PRAS40 would increase the phosphorylation level of the mTOR signaling pathway [36]. In contrast, other studies have determined that PRAS40 is essential to activate the mTOR signaling pathway [37] and is an important bridge linking Akt and mTOR signaling pathways [19]. In order to confirm the interaction between PRAS40 and mTOR signaling pathways in neuronal I/R injury, we used lentivirus-expressing PRAS40 to testify the role of PRAS40. It revealed that PRAS40 gene transfer promoted the phosphorylation of proteins in the mTOR signaling pathway, including mTOR, S6K, and 4E-BP1. In addition, PRAS40 overexpression reduced the release of LDH, which referred to ameliorating the I/R injury of primary cultured neurons. But the loss of PRAS40, knockdown by PRAS40 shRNA, showed an exact opposite trend. It inhibited the phosphorylation of proteins in the mTOR signaling pathway and aggravated the neuronal injury induced by OGD and reperfusion. Thus, our data support that PRAS40 is essential for mTOR activation and its downstream effectors in neuronal I/R injury.

Further experiments were performed to determine whether the protective effects of aescin was actually mediated by the PRAS40/mTOR signaling pathway. In this study, we applied aescin simultaneously with PRAS40 shRNA to investigate how PRAS40 mediated the biological function of aescin. Our results indicated that PRAS40 knockdown would lead to an increase of LDH release and undermine or even investigate how PRAS40 mediated the biological function of aescin against neuronal I/R injury in vitro. The results might contribute to the potential clinical application of aescin and provide a potential therapeutic target on I/R injury after endovascular thrombectomy or intravenous thrombolysis.

5. Conclusions

Aescin treatment is capable to protect primary cultured neurons against I/R injury in vitro. The PRAS40/mTOR signaling pathway is necessary for the protective effects of aescin against neuronal I/R injury in vitro. The results might contribute to the potential clinical application of aescin and provide a potential therapeutic target on I/R injury after endovascular thrombectomy or intravenous thrombolysis.

Data Availability

The raw data supporting the findings of this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors have declared no conflict of interest exists.

Authors’ Contributions

Xinjie Gao and Heng Yang contributed equally to this article.

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