Journal of the Neurological Sciences 339 (2014) 144–148

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Downregulated RASD1 and upregulated miR-375 are involved in protective effects of calycosin on cerebral ischemia/reperfusion rats

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ARTICLE INFO

Article history:
Received 11 December 2013
Received in revised form 19 January 2014
Accepted 3 February 2014
Available online 11 February 2014

Keywords:
Calycosin
ER-α
miR-375
RASD1
Apoptosis
Ischemia/reperfusion

ABSTRACT

Isoflavone calycosin is a typical phytoestrogen extracted from Chinese medical herb *Radix Astragali.* It has been reported that estrogens could provide neuroprotective effects, and dietary intake of phytoestrogens could reduce stroke injury in cerebral ischemia/reperfusion (I/R) animal models. In the present study, we investigate the molecular mechanisms underlying the neuroprotective effects of calycosin on middle cerebral artery occlusion (MCAO) rats. Focal cerebral ischemia was induced in male rats by MCAO, neurological deficits and brain edema was evaluated after 24 h of reperfusion. The results shown calycosin significantly reduced the infarcted volume and the brain water content, and improved the neurological deficit. To provide insight into the functions of estrogen receptor (ER)-mediated signaling pathway in neuroprotection by calycosin, the expression of miR-375, ER-α, RASD1 (Dexamethasone-induced Ras-related protein 1) and Bcl-2 was determined by RT-PCR or western blot assay. Calycosin exhibited a downregulation of RASD1, and an upregulation of ER-α, miR-375 and Bcl-2. Our finding illustrated that calycosin had been shown neuroprotective effects in cerebral ischemia/reperfusion rats, and the molecular mechanisms may correlate with the positive feedback between ER-α and miR-375, along with the regulation of downstream targets.

1. Introduction

Stroke, also called cerebrovascular accident, is the third leading cause of death in developed countries [1] and the most common reason for permanent disability [2]. And it is considered that cerebral ischemia/reperfusion (I/R) can lead to aggravated brain injury in the development of stroke and other cardiovascular diseases, which is induced by generation of reactive oxygen species [3], phospholipase activation [4,5], energy failure [6], inflammatory response [7,8] and programmed cell death [9,10], *etc.*

In recent years, data from different studies support the idea that estrogens provide neuroprotective effects in a variety of cerebral ischemia animal models [11–13]. However, due to the undesirable side effects of estrogen therapy (such as increased risk of cancers and irregular bleeding), phytoestrogens have received considerable attention as an alternative [14,15]. Phytoestrogens are estrogen-like molecules widely found in many natural products, especially soy. Because of their structural similarity with estrogen and ability to selectively bind estrogen receptors (ERs), phytoestrogens can produce estrogenic and/or anti-estrogenic effects [16]. In recent years, phytoestrogens have been intensively investigated as potential protective treatment in many diseases [17,18]. For example, it is reported that dietary intake of phytoestrogens could reduce stroke injury in animal model of focal and global cerebral ischemia [19–21]. However, an understanding of the underlying protection mediated by phytoestrogens from ischemic injury remains incomplete [22].

As a soy isoflavone phytoestrogen, calycosin ($C_{16}H_{12}O_5$) is proven to have properties of antioxidant, antivirus and apoptosis-modulation [23–25]. In our previous study, it is suggested that low concentrations of calycosin (<16 μM) had stimulatory effects on the proliferation of ER-positive cells *in vitro* and *in vivo* [26], which might be related to the expression of ERα (a subtype of ER) [27]. Next, we confirmed that, at relative low concentrations, calycosin upregulated the level of miR-375 and downregulated the level of Dexamethasone-induced Ras-related protein 1 (RASD1) protein in nerve cells. Notably, it has been reported that there exists a positive feedback regulation between miR-375 and ERα in breast cancer [28]. These results, combined with our previous findings, make us hypothesize that the positive feedback loop of miRNA-375/ERα and RASD1 may also be involved in the proliferative effect induced by calycosin in ERα-positive cells. Thereby, here we focused on the relationship between miRNA-375 and ERα, as well as their possible downstream effectors.

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http://dx.doi.org/10.1016/j.jns.2014.02.002

2. Material and methods

2.1. Drugs and animals

Calycosin (purity > 98%, verified by high performance liquid chromatography; purchased from Phytomarker, China) was dissolved in dimethyl sulfoxide (DMSO) to make a 200 mM stock solution and stored at 4 °C for further use. All Sprague–Dawley rats (male, 225–285 g, Grade II, No. 130217) were supplied by the Experimental Animal Center of Guilin Medical University. All the experimental procedures were performed in accordance with the guidelines of the Experimental Research Institute of Guilin Medical University.

2.2. Focal cerebral ischemia–reperfusion model

Rats were randomly divided into sham-operated, I/R and calycosin groups. The calycosin groups were respectively injected intraperitoneally with high-dose (20 $mg \cdot kg \cdot d^{-1}$), medium-dose (10 $mg \cdot kg \cdot d^{-1}$) and low-dose calycosin (5 $mg \cdot kg \cdot d^{-1}$) for 14 days. All rats were anesthetized with 10% chloral hydrate 1 h after the last drug administration. After skin incision, the right common carotid artery and external carotid artery were exposed. Then a nylon monofilament with a round tip was inserted into the external carotid artery and advanced into the internal carotid artery, until mild resistance was felt. Such resistance indicated that the filament had reached the circle of Willis. During these procedures, the rectal temperature was maintained at 37–38 °C with a heating lamp and heating pad. After ischemia for 2 h, the filament was gently removed and the animals were allowed to recover. While for the sham-operated group, rats underwent a neck dissection and surgical preparation of the external carotid artery, but not insertion of filament.

2.3. Neurological evaluation and infarct volume measurement

After ischemia (2 h), followed by reperfusion (24 h), rates were evaluated for neurological deficits using a 5-point scale system previously described [29]. Then rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (350 $mg \cdot kg^{-1}$). Brains were carefully removed and sliced to 2 mm thickness. The slices were stained with TTC for 30 min at 37 °C. The infarct areas were photographed on line, as previously depicted in detail [30]. Image analysis software (NIH Image, National Institutes of Health, Bethesda, version 1.63) was used for the measurement of the infarct areas.

2.4. Brain edema measurement

After 24 h of reperfusion, rats were decapitated and brains were immediately removed from the skull. Each hemisphere was weighed (wet weight), dried at 160 °C heater and then reweighed (dry weight). The degree of brain water content was calculated by the following equation: water content = (wet weight − dry weight) / wet weight × 100%.

2.5. RT-PCR

The ischemic regions of right cerebral cortices were collected and quickly frozen in liquid nitrogen. Then the tissues were transferred into Trizol (Gibco-BRL, USA) in a 1.5 mL tube for 15 min. Chloroform was added to the tube, shaken vigorously and then incubated for 10 min. The reaction tube was centrifuged at 12,000 *g* for 15 min at 4 °C. The upper portion was transferred into another tube. An equal volume of isopropyl alcohol was added, shaken and incubated for 10 min. The mixture was centrifuged at 12,000 *g* for 10 min at 4 °C. The RNA pellet was washed with ice-cold 75% ethanol, and centrifuged at 7500 *g* for 5 min at 4 °C. The RNA pellet was air-dried, then dissolved into RNase-free dH_2O. RNA was stored at −80 °C for further use. Total RNA was reverse transcribed with the RevertAid™, First Strand cDNA Synthesis Kit (Fermentas, USA) following the manufacturer's instructions. PCR products were separated on 1% agarose gel stained with ethidium bromide and observed under ultraviolet light.

2.6. Western blot analysis

The ischemic regions of right cerebral cortices were prepared in ice-cold lysis buffer. The lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and protein concentrations in the resulting supernatants were determined by Bio-Rad assay kit. Equal amounts of protein (40 μg/lane) were separated by SDS-PAGE and blotted onto 0.22 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, USA). The membranes were blocked in TBST (Tris-buffered solution, pH 7.6, 0.05% Tween 20) containing 5% nonfat dried milk at 4 °C overnight. Membrane were further incubated sequentially with primary antibodies including, RASD1 (1:1000), and β-actin (1:1000). After three washes, the blots were subsequently incubated with appropriate secondary antibodies coupled to horseradish peroxidase at room temperature for 1 h and then developed in electrochemiluminescence (ECL) western blot detection reagents (Beyotime, China).

2.7. Statistics

Data were expressed as mean ± standard deviation. The Statistical Package for Social Sciences (SPSS) 13.0 software (SPSS, Chicago, IL) was used for statistical analyses including one-way ANOVA and Student's *t*-test. A probability (p)-value <0.05 was considered statistically significant.

3. Results

3.1. Calycosin protected brain function from ischemia/reperfusion injury

As shown in Table 1, compared with I/R group, calycosin markedly improved neurological function, which manifested as decreased neurological score ($p < 0.01$). Accordingly, we found that the brain water content was significantly reduced with treatment of calycosin ($p < 0.01$) (Fig. 1B). When the brain was stained with TTC, infarct tissue was visualized as an area of unstained part in ischemia/reperfusion rats, whereas stained red in viable tissue. As expected, there was no infarct part in sham-operated group, and the infarct volume decreased in calycosin groups ($p < 0.01$), as shown in Fig. 1A. In addition, calycosin was shown to act in a dose-dependent manner to protect neuron cells from ischemia/reperfusion injury.

3.2. Effects of calycosin on miR-375, ER-α, RASD1 and Bcl-2 mRNA expression in cerebral ischemia/reperfusion rats

As shown in Fig. 2, more RASD1 and less Bcl-2 mRNA expression were observed in I/R group compared with those in sham group ($p < 0.01$). And with the treatment of calycosin, there was a gradual decrease in the RASD1 mRNA expression and a progressive increase in Bcl-2.

Table 1
Effect of calycosin on neurological score in cerebral ischemia/reperfusion (I/R) rats ($\bar{x} \pm SD$, $n = 8$).

Group	Dose ($mg \cdot kg^{-1}$)	Neurological score
Sham		0.0 ± 0.00
I/R		3.2 ± 0.69
Calycosin (high)	20	1.7 ± 0.52*
Calycosin (medium)	10	2.4 ± 0.79*
Calycosin (low)	5	2.9 ± 1.46

Neurological deficits were evaluated by EZ Longa Score Scale. Data are shown as the mean ± SD.
* $p < 0.01$ vs ischemia/reperfusion (I/R) group, $n = 8$ in each group.