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Full Length Article

Neuroprotective effects of pinocembrin on ischemia/reperfusion-induced brain injury by inhibiting autophagy

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ABSTRACT

Background: Cerebral ischemia/reperfusion (I/R) injury is a common pathological process after cardiac arrest, shock and acute cerebral infarction recanalization, which causes serious injury in brain function. Pinocembrin (Pino), a natural flavonoid at the highest concentration in propolis, exhibited a variety of biological effects, including antitumor, antimicrobial and anti-inflammatory activities. However, the effects of Pino on brain injured after I/R and the mechanisms of its neuroprotective effects remain elusive.

Methods: In the present study, we used I/R model rats underwent transient cerebral ischemia inducing by four-vessel occlusion and reperfusion. Pino alone or in combination with autophagy inducer rapamycin (RAPA) was administered to I/R rats. The behavior and cognitive function were evaluated by open field test and Morris water maze test. HE staining was used to determine the survival of hippocampus CA1 pyramidal cells. Three key proteins of autophagy, LC3, Beclin1 and p62, were detected by Western blot.

Results: Our results showed that Pino could significantly reduce the damage of hippocampus CA1 pyramidal neurons and alleviate the impairments of behavior and cognitive function in I/R rats. Pino also decreased the expression of LC3II and Beclin1 and increased the level of p62 in hippocampus CA1 of I/R rats. In addition, Pino also decreased RAPA-induced neuronal damage and excessive activation of autophagy in I/R rats.

Conclusions: Taken together, these results suggested that Pino could protect the brain injury induced by I/R and the potential mechanisms might attribute to inhibition of autophagy activity.

1. Introduction

Ischemic cerebrovascular abnormality is a kind of common and severe health-threatening disorder with high rates of morbidity, mortality and permanent disability in the central nervous system [1–3]. Cerebral ischemia is caused by insufficient blood supply to local area of brain, which in turn triggers various pathophysiological changes. Thrombolysis therapy has been employed to deal with ischemic cerebrovascular abnormality. After thrombolysis, the blood reperfusion will lead to a more serious tissue injury, called I/R injury [4,5]. Many studies have reported that energy metabolism deficiency, glutamate/neurotoxin release, inflammation, oxidative stress, and apoptosis may be occurred in the I/R brain injury [6–8].

Pinocembrin (5,7-dihydroxyflavanone, Pino), a natural flavonoid in the highest concentrations in propolis, has caught much attention for its various biological effects on anti-inflammatory, anti-oxidant, anti-microbial and anti-apoptotic activities [9–11]. Finding from previous studies showed that pinocembrin protected against ischemic injury and reduced the area of cerebral infarction in ischemia models [12]. Pino can improve cognition impairments of chronic cerebral hypoperfusion by its protections on brain mitochondria structure and function [13,14]. In addition, Pino also has a neuroprotective effect on the brain from I/R rats, but the mechanisms of its neuroprotective effects remain unknown [15,16]. The damage of primary cultured cortical neurons and cerebral microvessel endothelial cells in oxygen-glucose deprivation/reoxygenation could be alleviate by Pino [17].

It has been reported that autophagy can be induced by I/R. Autophagy is a catabolic process to eliminate cytoplasmic material through lysosomal degradation, which is essential for maintaining cellular homeostasis and survival. Mechanism of autophagy relies on the activity of various members of autophagy-related (ATG) protein family. LG3 can be lapidated by catalyzing of ATG3, ATG4 and ATG7,

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subsequently gathers and binds to autophagosomal membranes, to form LC3-II, which is an important marker of autophagy [18]. Besides, the protein p62 interacting with LC3 on autophagosomes is degraded through the autophagy–lysosome system [19]. Beclin1, autophagy marker, can regulate the process of autophagy by recruiting other proteins for the formation of autophagosome [20]. Some studies have indicated that autophagy connected with several diseases such as neurodegeneration in the central nervous system and neuronal death in cerebral ischemia and hypoxia [21–23]. Autophagy associates with regulating neuronal cell death in different models [24]. Excessive autophagy contributes to neuron death in cerebral ischemia [25]. Accumulating evidence indicated that Autophagy is an important regulatory pathway for I/R injury [26–28].

However, little is known about the neuroprotective effects of Pino on I/R-Induced brain injury and the neuroprotective mechanism of Pino related to autophagy. The present study employed pharmacological, histochemistry and Western blot techniques to investigate the impact of Pino on the neuroprotection in I/R rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (6–8 weeks, 240 ± 10 g) were supported from Animal Experiment Center of Tongji University of China. Rats were raised in standard housing conditions (room temperature: 25 ± 1 °C and humidity: 55–65%) under a 12-h light/dark cycle. Food and water were available ad libitum. All experiments were carried out according to the Provision and General Recommendation of the Chinese Laboratory Association.

2.2. Drugs and antibodies

Pino (P5239) was purchased from Sigma-Aldrich (Saint Louis, USA). RAPA (S1842) was obtained from the Beyotime Biotechnology (Shanghai, China). The antibodies p62 (sc-25575) and Beclin1 (sc-48341) were purchased from Santa Cruz Biotechnology (Dallas, USA). Anti-LC3 (#12741) was obtained from Cell Signaling Biotechnology (Danvers, USA).

2.3. I/R model establishment

Rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and implanted with stainless steel cannulae (26 gauge) into the right lateral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral 1.5 mm; depth 3.5 mm from the skull surface). After correct positioning, cannulae were fixed to the skull with cranioplastic cement and sealed with 30-gauge dummy cannulae. Transient cerebral ischemia was induced through 4-vessel occlusion [29,30]. Rats received a recovery period of 24 h and fasted overnight. On the following day, both common carotid arteries were occluded with aneurysm clips to induce cerebral ischemia. And the aneurysm clips were removed for reperfusion after 15 min of the occlusion. Rats that lost their righting reflex and whose pupils were dilated and unresponsive to light were chosen for the present study. A rectal thermometer was inserted to monitor body temperature. Additionally, another needle thermometer positioned subcutaneously on the temporal muscle was used to monitor the epidural temperature. Both temperatures were maintained at 36.5–37.5 °C with a filament lamp combined with an Automated Thermal Control Unit (DC Temperature Controller, FHS 40-90-8D, Bowdoin, ME) throughout the procedures. Rats with seizures were excluded. Control groups were performed using the same surgical procedures except that the carotid arteries were not occluded. In the present study, 130 rats were performed the I/R model operation, in which 112 rats were successfully treated, and 3 rats were died. All 35 rats in control groups were alive.

2.4. Administration of drug

The rats were treated with Pino (5 mg/kg in saline) was administrated intravenously 30 min before ischemia [31]. RAPA (10 μL, 0.01 mol/L in DMSO) administered by injection in the right lateral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral 1.5 mm; depth 3.5 mm from the skull surface) 30 min before ischemia on the first day [32]. After 24 h of reperfusion, Pino and RAPA were administrated again. The rats continued to be administered pinocembrin or the vehicle intravenously once a day at the same time until they were sacrificed. The control group and I/R group rats were injected with the same volume of vehicle. During the behavioral test, the drug was administered 40 min before the trials.

2.5. Experimental design

147 rats were randomly divided into two groups, group A and group B. Rats in group A were further divided into three groups, which were control group (without I/R),I/R (I/R and normal saline treatment) group and I/R + Pino group. Daily administration of Pino and terminated on day of killing [16]. Each subgroup consists of 14 rats. In group B, rats were further divided into five groups, namely control group, I/R (I/R and normal saline treatment) group, I/R + Pino (I/R and Pino treatment) group, I/R + RAPA (I/R and Rapa treatment) group and I/R + RAPA + Pino (I/R with Rapa and Pino treatment). Each subgroup consists of 21 rats (Table1).

2.6. Open field testing

After reperfusion for 7 days, the locomotor activities and exploratory behaviors of rats were conducted in an open field. The open field device consists of an opaque acrylic container, a square and a video camera which fixed 1 m above the area tracking the rats' movement. Using the computerized tracking system to analyze the images and measure speed and distance of rats' movement. Rats were put into the dimly lit room for 1 h to adapt to the new environment before testing. Then the individual rat was placed on the middle of the chamber for each trial. The rat was then free to move around in the open field for 20 min. During the test, the grooming sessions, the number of rearing events, total distance and speed traveled were recorded. Besides, the time spent in the central area of the open field was also recorded to assess the anxiety of rats.

2.7. Morris water maze test

The Morris water maze (MWM) test was performed to assess the spatial learning and memory of the rats after the open field testing, as

Table 1
Experimental groups and the number of rats in each group.

Group	Subgroup	Rat number	Experiment	Rat number per experiment
1	Con	14	MWM,HE,OFT,	7
1	I/R 7d	14	MWM,HE,OFT,	7
1	I/R 7d + Pino	14	MWM,HE,OFT,	7
2	Con	21	MWM,HE,OFT,WB	7
2	I/R 7d	21	MWM,HE,OFT,WB	7
2	I/R 7d + Pino	21	MWM,HE,OFT,WB	7
2	I/R 7d + RAPA	21	MWM,HE,OFT,WB	7
2	I/R 7d + RAPA + Pino	21	MWM,HE,OFT,WB	7

I/R: ischemia/reperfusion; Pino: Pinocembrin; RAPA: Rapamyein.
MWM: Morris water maze testing.
HE: hematoxylin-eosin staining.
OFT: Open field testing.
WB: Western blotting.

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