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The characteristics of therapeutic effect of pinocembrin in transient global brain ischemia/reperfusion rats

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ABSTRACT

Aims: The therapeutic effect of pinocembrin, together with the therapeutic time window, in the transient global cerebral ischemia/reperfusion (I/R) rats was investigated.
Main methods: Adult male Sprague–Dawley rats were subjected to global cerebral ischemia for 20 min by four-vessel occlusion. Pinocembrin (1 and 5 mg/kg) was administrated intravenously 30 min before ischemia and 30 min, 2 h, 6 h after reperfusion, respectively. Neurological scores, brain edema and histological examination by Nissl staining were employed to assess the neuronal injury after ischemia and the neuroprotection by pinocembrin. The activities of superoxide dismutase (SOD), myeloperoxidase (MPO) and the content of malondialdehyde (MDA) in brain tissue were tested by colorimetric assays. Alterations of neurotransmitters were determined by a high performance liquid chromatography–electrochemical method.
Key findings: Pinocembrin significantly ameliorated neurological deficits and brain edema, and alleviated the degree of hippocampal neuronal loss at 24 h after global cerebral I/R with a broad therapeutic time window. It was found that treatment with pinocembrin reduced the compensatory increase of SOD activity and decreased the MDA level and MPO activity in a dose-dependent manner. The metabolic balance between excitatory and inhibitory amino acids was modulated by pinocembrin treatment.
Significance: These findings suggest that pinocembrin provides neuroprotection against global cerebral ischemic injury with a wide therapeutic time window, which may be attributed to its antioxidative, antiinflammatory and antiexcitotoxic effects.

Introduction

Transient global cerebral ischemia is one of the devastating events that can occur with great morbidity, during asphyxiation, shock, brain injury, extracorporeal circulation and cardiac arrest (Weigl et al. 2005). Cerebral ischemia is a result of insufficient blood supply to a section of the brain, which in turn triggers various pathophysiological changes (Mehta et al. 2007). The central event during cerebral ischemia/reperfusion (I/R) is the generation of reactive oxygen species (ROS) which lead to neuronal death, brain edema and inflammation (Moro et al. 2005). Abundant evidence also demonstrates that the imbalance between excitatory and inhibitory amino acids play a crucial role in the brain injury sustained by I/R (Dirnagl et al. 1999; Hazell 2007; Mehta et al. 2007).

Pinocembrin (5,7-dihydroxyflavanone) is one of the flavonoids present in the highest concentrations in propolis (Bankova et al. 1982). Pinocembrin exhibits multiple biological actions such as antimicrobial (Park et al. 1998; Uzel et al. 2005), vasorelaxative (Zhu et al. 2007), antioxidant (Sala et al. 2003; Santos et al. 1998), antiinflammatory (Habtemariam 1997; Sala et al. 2003) and anti-apoptotic activities (Liu et al. 2008; Santos et al. 1998). Recently, several research projects on this compound have been undertaken in our laboratory. Findings from these projects have indicated that pinocembrin has the potential to reduce oxygen glucose deprivation induced injury in SH-SY5Y neuronal cells and primary cultured cortical neurons as well as reduce ischemic injury in middle cerebral artery occlusion (MCAO) rats. These potential reductions in injury are attributed to pinocembrin's antioxidative and antiapoptotic effects (Gao et al. 2008a, 2008b; Liu et al. 2008). However, there are no reports on the therapeutic potential of pinocembrin for global cerebral I/R. In the present experiment, the transient global cerebral I/R model of rats, induced by four-vessel occlusion (4-VO), was employed to evaluate the neuroprotective effect and the therapeutic time window of pinocembrin.

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Materials and methods

Animals

Male Sprague–Dawley rats weighing 300–350 g were obtained from Vital River Experimental Animal and Technology Co., Ltd (Certificate No. SCXK 2007–0001, Beijing, China). The rats were housed in an air-conditioned room (T = 21 ± 3 °C) and exposed to a natural light/dark (12/12 h) cycle. The rats were allowed free access to pellett food and water. All procedures were performed in accordance with the institutional guidelines for laboratory animals and were approved by the Animal Care Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences. Every effort was made to reduce suffering and minimize the number of animals required.

Administration of pinocembrin

Pinocembrin was first isolated by the Department of Natural Product Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences. Pinocembrin used in the present study was synthesized and processed using a sterile injection powder (hydroxypropyl-beta-cyclodextrin). It was then dissolved in physiological saline before use. The rats were randomly assigned into 4 groups: sham group (physiological saline); vehicle group (hydroxypropyl-beta-cyclodextrin); pinocembrin-treated groups (1 and 5 mg/kg). All drugs were injected via the tail vein 30 min before ischemia and 30 min, 2 h and 6 h after reperfusion.

Surgical procedure

The animals were anesthetized with 400 mg/kg chloral hydrate (i.p.). In brief, after the animals were positioned in the rat brain stereotaxic apparatus, an incision of 1 cm in length was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed. A 0.5-mm-diameter electrocautery needle was inserted through each alar foramen and both vertebral arteries were electrocarterized and permanently occluded. Next, both common carotid arteries were isolated via a ventral midline cervical incision. A suture was loosely placed around each common carotid artery without interrupting carotid blood flow. On the following day, rats were re-anesthetized with ether to open the wound. Both carotid arteries were then occluded with microvessel clips for 20 min. After 20 min, the clips were removed for reperfusion. To minimize variability among animals, only the rats that lost righting reflex with gray eyes during the entire ischemic period were included in the study (Ma et al. 2008). Sham operated rats were treated similarly to the ischemic group without occlusion of the four vessels.

Assessment of neurological scores

After the completion of the I/R period and before their sacrifice, the animals were assessed for neurobehavioral activity when they came back into consciousness. Performance was scored by a 25-point scale (Table 1) modified from the previous literature (LeMay et al. 1988; Sun et al. 2009; Xu et al., 2001).

Table 1
Neurological deficit score for rats.

Symptoms	Scores
Hair roughed up	1
Decreasing movement or bradypragia	1
Enhancement of response to ear-palpating	1
Eyelid ptosis	1
Upwarping head or hunched posture	3
Eye misclosure or patency	3
Circling behavior	3
Splayed-out hind limb	3
Hyperspasmia or clonus	3
Myasthenia of limbs	6
Total scores	25*

* Mild ischemia <10, severe ischemia ≥10.

Preparation of cerebral homogenate

The rats were killed by decapitation, 24 h after cerebral I/R. The brains were then quickly removed, weighed and sliced in half. The left hemispheres of the brains were used for evaluating brain edema, while the right brain hemispheres were homogenized in 5 volumes (w/v) of buffer (Tris–HCl 50 mM, NaCl 150 mM, $CaCl_2$ 5 mM, PMSF 0.1 mM, pH 7.4) on ice and then centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatant of each sample was obtained and stored at −80 °C until analysis.

Determination of superoxide dismutase (SOD), myeloperoxidase (MPO) activities and malondialdehyde (MDA) content

SOD, MPO activities and MDA content were assessed by using SOD, MPO and MDA detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the methods described by the assay kits. Briefly, SOD activity was determined using xanthine oxidase method (Ahmad et al. 2006). Tissue homogenate was mixed with reagents supplied by the manufacturer and maintained at 37 °C for 40 min. The absorbance at 550 nm was measured after 10-min incubation at room temperature with developer. For MPO, the measurement was based on the enzyme's ability to degrade H_2O_2 which reacts with guaiacol to show absorbance at 460 nm (Miljkovic-Lolic et al. 2003). MDA content was detected by modified thiobarbituric acid method (Ohkawa et al. 1979). Brain homogenate was mixed with dehydrated alcohol, thiobarbituric acid and developer. It was then maintained in a water bath at 95 °C for 40 min, and centrifuged at 3000 r/min for 15 min. The absorbance of the supernatant was determined at 532 nm. The SOD and MPO activities were expressed as units/g protein. A SOD unit was defined as the amount of protein required to decrease the reference rate to 50% of maximum inhibition. A MPO unit was defined as the amount of enzyme required to degrade 1 μM H_2O_2 in the reaction system. The MDA concentration was expressed as nmol/g protein.

Evaluation of brain edema

Following decapitation 24 h after reperfusion, the right brain hemispheres were obtained as described above and weighed immediately to obtain the wet weight. The tissue was then dried in an oven at 120 °C for 24 h and then reweighed to obtain the dry weight. Cerebral water content was calculated according to the following formula:

$$[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%.$$

Histopathological analysis

24 h after reperfusion the rats were deeply anesthetized with 400 mg/kg chloral hydrate (i.p.) and their brains were perfused with 100 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde (pH 7.4). The brains were then removed and postfixed in the same fixative for 3 days at room temperature, and embedded in paraffin. 5 μm-thick coronal sections were cut and mounted onto poly-L-lysine coated slides. Nissl staining (cresyl violet) was performed for different groups according to the standard histochemical procedure. Since CA1 is especially vulnerable to I/R injury (Crain et al. 1988), the CA1 area was selected as the target area to be observed. The number of normal