Journal of Ethnopharmacology 144 (2012) 313–321

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Ligustilide alleviates brain damage and improves cognitive function in rats of chronic cerebral hypoperfusion

Zhanbo Feng, Yapeng Lu, Xiaomei Wu, Peng Zhao, Jiejia Li, Bin Peng, Zhongming Qian, Li Zhu *

Department of Neurochemistry, Institute for Nautical Medicine, Nantong University, Nantong 226001, China

ARTICLE INFO

Article history:
Received 21 March 2012
Received in revised form
24 July 2012
Accepted 3 September 2012
Available online 27 September 2012

Keywords:
Ligustilide
Chronic cerebral hypoperfusion
Morris water maze
Neuron
Astrocyte
Hippocampus

ABSTRACT

Ethnopharmacological relevance: Ligustilide (LIG), a main lipophilic component of *Danggui* (Chinese Angelica root, *Radix Angelica sinensis*) which is a popular used herb to treat menstrual disorders in traditional chinese medicine, has been reported to possess some neuroprotective effects on permanent focal ischemia and transient forebrain ischemia.

Aim of the study: Based on previous work, we intended to investigate the protective effects of LIG on parietal cortex and hippocampus of rats in chronic cerebral hypoperfusion model.

Materials and methods: Chronic cerebral hypoperfusion was induced by permanent, bilateral common carotid artery's occlusion (2VO). The rats were treated with LIG (80 mg/kg, by oral) from the eighth day after surgery for seven consecutive days. Their spatial learning and memory abilities were assessed using the Morris water maze. After six days for maze test, rats were sacrificed. Coronal sections in cortex and hippocampus were stained with cresyl violet or labeled with NeuN (Neuronal Nuclei), MAP-2 (Microtubule-Associated Protein-2), Caspase-3 and GFAP (Glial Fibrillary Acidic Protein) antibodies.

Results: LIG treatment for seven days decreased escape latency and swimming distance of 2VO rats from the third day in maze tests, and increased percent time in the target quadrant. LIG prevented neuronal loss, dendrites damage and neuronal apoptosis in both parietal cortex and hippocampus of 2VO rats; and it also inhibited astrocytic activation and proliferation stimulated by hypoperfusion.

Conclusions: These results demonstrate that LIG show obvious neuroprotective potential for treating chronic cerebral hypoperfusion injury, which may be attributed to its anti-apoptosis of neuron and anti-proliferation of astrocyte both in cortex and in hippocampus of 2VO rats. We suggest that LIG can be developed as an effective drugs for the prevention of vascular dementia (VD).

1. Introduction

Danggui, known as *Radix Angelica sinensis* (the dried root of *Angelica Sinensis* (Oliv.) Diels (Umbelliferae)), is one of the most commonly used herbs in traditional chinese medicine. The ethnopharmacology, chemistry, pharmacology, pharmacokinetics, toxicity, and clinical applications of *Radix Angelica sinensis* are extensively reviewed (Sun, 2005; Tang et al., 2009; Zheng et al., 2010). In clinic, *Danggui* is officially listed in the Chinese Pharmacopoeia for the treatments of menstrual disorders, cardiovascular and cerebrovascular diseases. As the main lipophilic component of the essential oil constituents and a characteristic phthalide component of many Umbelliferae plants, Ligustilide (LIG) is mainly isolated and purified from *Danggui* (Dong et al., 2007; Li et al., 2006). The anti-thrombotic, antitoxic and anti-inflammatory effects of LIG in nervous system have been discovered (Zhang et al., 2009; Wang et al., 2010; Shao et al., 2011). In pharmacokinetic studies of LIG, it is detected in rat brain after 5–20 min of nasal and oral administration, indicating that LIG has a rapid onset of action to enter the central nervous system by permeating blood–brain barrier (Guo et al., 2011). In various studies in vitro, LIG has been shown many effects on inhibiting uterine contraction, reducing acetic acid-induced writhing response and formalin-induced licking time, and inducing vasodilatation in rat aorta and mesenteric artery (Cao et al., 2006; Du et al., 2006; Chan et al., 2007; Du et al., 2007). Moreover, LIG protects against hydrogen peroxide-induced injury in PC12 cell in vitro through antioxidant and anti-apoptotic mechanisms (Yu et al., 2008).

LIG reduces the infarct volume in transient forebrain ischemia in mouse and protects against ischemia-reperfusion injury in rats (Kuang et al., 2006; Wu et al., 2011). Similar neuroprotective effects of LIG have been found in the rat of focal cerebral ischemia injury induced by the occlusion of middle cerebral artery for 24 h (Peng et al., 2007). In permanent forebrain ischemia rat, LIG ameliorates cognitive dysfunction and hippocampal damage for 28-days treatment (Kuang et al., 2008). Besides, LIG protects

Abbreviations: CA1, the CA1 area of the hippocampus; CBF, Cerebral Blood Flow; ChaT, Choline Acetyl Transferase; DAB, Diaminobenzidine; GFAP, Glial Fibrillary Acidic Protein; HPLC, High Performance Liquid Chromatography; LIG, Ligustilide; MAP-2, Microtubule-associated protein-2; NeuN, Neuronal Nuclei; PBS, Phosphate-Buffered Saline; PC, Parietal Cortex; 2VO, Bilateral Common Carotid Artery Occlusion (two-vessel occlusion); VD, Vascular Dementia

* Corresponding author. Tel.: +86 513 85051796; Mob.: +86 13962988532; fax: +86 513 85051796.
E-mail address: zhulili65@126.com (L. Zhu).

0378-8741/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.09.014

against the amyloid β-peptide induced neurotoxicity by inhibiting TNF-α-activated NF-κB signaling path (Kuang et al., 2009).

Various experimental and clinical observations suggest that chronic cerebral hypoperfusion is associated with learning and memory dysfunction and contributes to neurological diseases, such as vascular dementia (VD) (Annahazi et al., 2007; Farkas et al., 2007; Institoris et al., 2007). Permanent, bilateral common carotid artery's occlusion (2VO) of rat has been introduced to reproduce chronic cerebral hypoperfusion as it occurs in VD (Farkas et al., 2007). In 2VO rats, persistent reduction of cerebral blood flow (CBF) in cortex, white matter, and hippocampus leads to cerebral hypoperfusion and thus induces suboptimal metabolism, cognitive impairment, and neuronal injury (Otori et al., 2003). Several studies based on this model have been performed to explore some potentially beneficial medicines and strategies to delay the dementia processes (He et al., 2009; Lee et al., 2009; Cai et al., 2011).

Moreover, chronic cerebral hypoperfusion leads neuronal damage not only in the hippocampus but also in the cortex (Shibata et al., 2004; Cho et al., 2006; Ueno et al., 2009). In present research, we observed the protective effects of LIG on both parietal cortex and hippocampus of 2VO rats. We analyzed the changes in the learning performance, neuronal damage, dendritic integrity, neuron apoptosis and astrocytic activation. The results show that LIG treated for one week partially protect neruons in parietal cortex and hippocampus against 2VO induced brain damage and cognitive dysfunction.

2. Material and methods

2.1. Animals and surgical procedures

Male Sprague-Dawley rats (260 g ± 10 g) were purchased from Experimental Animal Center of Nantong University and were used after one week of quarantine and acclimatization. Forty five rats were induced by bilateral common carotid artery's occlusion as 2VO models; fifteen rats served as sham-operated controls (SHAM group). The rats were anesthetized with 350 mg/kg chloral hydrate intraperitoneally. Following a midline incision, the bilateral common carotid arteries were separated from the cervical sympathetic and vagal nerves, and were ligated doubly with 4-0 silk suture (32 of 45 survivals). The SHAM group was suffered same operation without actual ligation. The animals were housed in three in stainless steel cages at 21 ± 2 °C and had free access to food and water. Rooms were in a cycle of 12 h of light (6:00 to 18:00) and 12 h of darkness (from 18:00 to 6:00). Our project was submitted to ethics committee on animal experimentation in Nantong University and all procedures were approved according to the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (Approval ID: SYXK(SU)2007-0021), which comply with international rules and policies.

2.2. LIG preparation and rat treatment protocol

Danggui was purchased from the Danggui Cultivating Base of Good Agricultural Practice in Nin Xian County, Gansu Province, PRC and LIG was prepared by a well established procedure as described previously (Qian et al., 2005; Wu et al., 2011). Its purity was over 98% based on the percentage of total peak area in the high performance liquid chromatography (HPLC) analysis. LIG (500 mg) was dissolved in soybean oil (10 mL) and mixed thoroughly.

On the eighth day after surgery, the survival rats were divided into 2VO, LIG and SHAM group. The LIG group was given LIG (80 mg/kg body weight per day) orally; the SHAM and 2VO group were given equal volume of soybean oil as the vehicle. The treatments were performed for seven consecutive days. The animals were assigned to behavioral testing on the next day after the final administration, and continued for the following five days.

2.3. Morris water maze test

On the fifteenth day following surgery, the spatial learning capacities of all rats were assessed by the Morris water maze as previous reports (Vorhees and Williams, 2006). The maze consists of a black circular pool and a hidden platform submerged 2 cm below the water (22 ± 1 °C) surface. The pool was signed into four quadrants as N (North), S (South), W (West), E (East) and the platform was located in the SW (Southwest). The rat was released randomly into the water at water-level facing the tank wall from N, E, SE and NW. The training trials, also called hidden platform trials, were tested four trials per day and conducted for five continuing days. A computer tracking program was started as soon as the rats were released into water, and stopped when the rats climbed on the platform or did not find the platform within 2 min. Some visual cues helping rats escape from water were placed on the wall of the testing room and the tank. The probe trials were performed at the next day when spatial training trials were finished. Platforms were removed, and the rats were placed in a novel start position in the maze, facing the tank wall 180°from the original platform position. The time for probe trials lasted 30 s. Percent time in the target quadrant was calculated. For all trials, escape latency, swimming distance and swimming paths were recorded by a computerized video imaging analyze system (Institute of Material Medical, Chinese Academy of Medical Sciences, China).

2.4. Histology and immunofluorescence

On the next day after the probe trials finished (the twenty-first day after the surgery), six rats of each group were anesthetized with an overdose of 10% chloral hydrate intraperitoneally and the brain slices were prepared for histological studies. Coronal sections were cut at 25 μm thickness by a frozen-stage microtome. The first sets of sections were mounted on polylysine-coated microscopic slides and stained with 1% cresyl violet. The second sets of sections were labeled by immunofluorescence or immunocytochemical method according to previously established protocols in our laboratory. Firstly, the sections were incubated with 3% H_2O_2 and goat blocking serum; secondly, the sections incubated with primary antibodies overnight at 4 °C [mouse anti-NeuN antibody (Millipore, 1:200, USA.), rabbit anti-MAP-2 antibody (Millipore, 1:200, USA.), rabbit anti-Caspase-3 antibody (Bioworld, 1:200, USA) and mouse anti-GFAP antibody (Millipore, 1:600, USA)]. For NeuN labeling, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H+L) (Bioworld, 1:200, USA) in PBS; for MAP-2 labeling, the sections were incubated with tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (H+L) (Bioworld, 1:200, USA) in PBS; for Caspase-3 labeling, the sections were incubated with tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (H+L) (Bioworld, 1:200, USA) and goat anti-rabbit polink-2 plus polymer detection systems (Zhongshan Golden Bridge, Beijing, China) in PBS; for GFAP labeling, the sections were incubated with goat anti-mouse polink-2 plus polymer detection systems (Zhongshan Golden Bridge, Beijing, China) in PBS. The sections which incubated with polink-2 plus polymer detection systems were conventionally developed with diaminobenzidine (DAB) and H_2O_2. Photographs of the hippocampus CA1 stratum pyramidal and parietal cortex were taken with a computerized image analysis system (Leica DM4000B microscope, Germany).

For Nissl staining and NeuN immunostaining, we counted the number of positive cells in the parietal cortex region, and the percentage surface areas (shown as the ratio of SHAM) in the hippocampus CA1 region (0.27 mm^2). For Caspase-3 and GFAP immunostaining, we counted the number of immunopositive cells in the parietal cortex and hippocampus (0.07 mm^2 for Caspase-3,