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Vitexin reverses the autophagy dysfunction to attenuate MCAO-induced cerebral ischemic stroke via mTOR/Ulk1 pathway

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ABSTRACT

Stroke, as a kind of acute cerebrovascular diseases, has greatly influenced the patients' quality of life and left a huge public health burden. Vitexin is a flavone C-glycoside (apigenin-8-C-β-D-glucopyranoside) present in several medicinal and other plants. This study aims to explore the role of vitexin in middle cerebral artery occlusion (MCAO)-induced cerebral ischemic stroke. The results showed that the MCAO-induced brain infarction was obviously decreased by vitexin. And the abnormal protein levels of Caspase-3, Bcl-2-associated X protein (Bax), antigen identified by monoclonal antibody (Ki-67) and B cell lymphoma 2 (Bcl-2) in MCAO model rats were reversed by vitexin. Further research indicated that vitexin alleviated MCAO-induced oxidative injury by reducing the levels of lactate dehydrogenase (LDH), malondialdehyde (MDA) and nitric Oxide (NO). In addition, vitexin attenuated the secretion of pro-inflammatory cytokine (interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α)) and increased anti-inflammatory cytokine (IL-10) production to ameliorate MCAO-induced inflammation. What's more, vitexin repressed the MCAO-induced autophagy through mechanistic target of rapamycin (mTOR)/Ulk1 pathway. Specifically, the MCAO-induced decreased expression of mTOR, peroxisome proliferator-activated receptor γ (PPARγ) and p62 were inhibited by vitexin. At the same time, MCAO-induced increased expression of Ulk1, Beclin1 and rate of LC3II/LC3I also were repressed by vitexin. But the inhibition of vitexin on the MCAO-induced oxidative injury, apoptosis and inflammation were reversed by rapamycin. These results implied that vitexin suppressed the autophagy dysfunction to attenuate MCAO-induced cerebral ischemic stroke via mTOR/Ulk1 pathway.

1. Introduction

Stroke is a medical condition in which angiorrhexis or angiemphraxis leads to poor blood flow to the brain. Stroke can be classified into two major pathological types: ischemic and hemorrhagic stroke. Ischemic stroke, also known as cerebral infarction, was the most common form of stroke and characterized by the brain tissue damage caused by occlusion of cerebral arteries. Ischemic stroke was considered as one of the major fatal diseases in the world with high incidence, mortality, disability and recurrence rates [1]. The burden of ischemic stroke increased significantly between 1990 and 2010 worldwide in terms of the absolute number of people with incident ischemic stroke. There were 11 million ischemic stroke cases in 2010 and 2 million individuals died from ischemic stroke [2]. So far, few therapeutic strategies are available for the treatment of ischemic stroke. It is urgent to explore effective therapies for ischemic stroke.

Chinese herbal medicines and other natural products, based on thousands years of clinical treatment and prevention experience, have many advantages to slow down or prevent disease progression. In the past decades, hundreds of compounds isolated from natural herbs or foods have been identified to ameliorate ischemic stroke, such as phenolic compounds, saponins, terpenoids and alkaloids [3]. Vitexin is a flavone C- glycoside (apigenin-8-C-β-D-glucopyranoside) present in several medicinal and other plants, such as hawthorn, passion flower, bamboo leaves and chasteberry [4]. It was reported that vitexin had recently received increased attention due to wide range of pharmacological effects, including but not limited to anti-oxidant, anti-inflammatory, anti-hyperalgesic and neuroprotective effects [5].

Abbreviations: MCAO, middle cerebral artery occlusion; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; Ki-67, identified by monoclonal antibody; LDH, lactate dehydrogenase; MDA, malondialdehyde; NO, nitric oxide; IL, interleukin; TNF-, αtumor necrosis factor alpha; mTOR, mechanistic target of rapamycin; PPARγ, peroxisome proliferator-activated receptor γ; LC3, protein 1 light chain 3

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Therefore, it is significant to study the role of vitexin in stroke.

Autophagy is a lysosome-mediated degradation process for non-essential or damaged cellular constituents, playing an important homeostatic role in cell survival, differentiation and development to maintain homeostasis [6]. Autophagy has been reported to be associated with many cardiovascular diseases and cerebrovascular diseases, including stroke, hypertension, atherosclerosis and cardiomyopathy [7]. It was reported that targeting autophagy might be a promising option in the treatment of cerebral ischemia [8]. The mammalian target of rapamycin (mTOR) is a key regulator of autophagy. Dysregulation of mTOR signaling is associated with cancer, diabetes, and autism [9]. Thus, it is need to exploit anti- autophagy medicines for patients with stroke.

Therefore, the purpose of this study is to investigate the role of vitexin in MCAO-induced cerebral ischemic stroke. The MCAO-induced brain infarction and apoptosis were decreased by vitexin. Besides, vitexin ameliorated MCAO-induced oxidative injury and inflammation. Moreover, MCAO-induced autophagy was mitigated by vitexin via mTOR/Ulk1 pathway.

2. Materials and methods

2.1. Middle cerebral artery occlusion (MCAO) model

A total of 20 male Sprague–Dawley (SD) rats weighing 250–300 g were purchased from the Institute of Zoology, Chinese Academy of Medical Sciences. Rats were randomly divided into four groups. Control group: healthy rats; Sham group: rats received surgery without MCAO; MCAO group: rats were subjected to MCAO; MCAO + vitexin: one hour before MCAO, rats were intravenously injected with vitexin (2 mg/Kg body weight) in saline. Rats were anesthetized with intra-peritoneal injection of 10% chloral hydrate (350 mg/kg) and subjected to MCAO as previously reported with minor modifications [10]. In short, the external carotid artery (ECA) was tied. A 4–0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd, Beijing, China) with a rounded tip was aseptically inserted from the right common carotid artery (CCA) to the internal carotid artery (ICA) through the stump of the ECA and gently advanced to occlude the left middle cerebral artery (MCA) at its origin (18–20 mm). Reperfusion was achieved by gently removing the monofilament until the tip cleared the lumen of the ECA after 1.5 h of occlusion. In sham-operated animals, all procedures except occlusion of the MCA were performed. Rectal temperature was monitored throughout the operation and maintained at 37 °C with a circulating heating pad. After recovery from anesthesia, rats were kept in their cages with free access to food and water. The process in our study was approved by Central Hospital of Hanzhong City. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.2. 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

At first, brains were carefully dissected 24 h after MACO. Coronal sections (2 mm) were cut in a pre-cooled stainless matrix. Then, sections were placed into 24-well plates and incubated with 2% TTC stain in saline at 37 °C for 15 min. Gentle stirring ensured even staining exposure. Excess TTC was drained off, and slices were fixed in

10% neutral buffered formalin. TTC stains viable brain tissue deep red but infarcted tissues keep the original pale color. Infarct volume and total brain volume were quantified with an Image J analysis system. Brain infarct volumes were expressed as the percentage of infarcted tissue relative to total brain tissue.

2.3. Western blot

The brain tissues were homogenized in cell lysis buffer (Tissue Protein Extraction Reagent; Thermo Scientific, Kanagawa, Japan) added with complete protease inhibitor cocktail (Roche). Equal amounts of protein in each sample were separated by SDS-PAGE. Then proteins were transfered from the gel to the Polyvinylidene Fluoride (PVDF) membranes (Millipore, Massachusetts, USA) for immunoblotting. After blocking, the membranes were incubated successively with primary antibodies at 4 °C overnight. The following primary antibodies (Abcam, Cambridge, MA, USA) were used: anti-Ki67 (1:5000 dilution), anti-Caspase3 (1:500 dilution), anti-Bax (1:1000 dilution), anti-Bcl-2 (1:2000 dilution), anti-mTOR (1:2000 dilution), anti-Ulk1 (1:1000 dilution), anti-PPAR-γ (1:1000 dilution), anti-Beclin1 (1:1000 dilution), anti-p62 (1:1000 dilution) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000 dilution), which was used as the internal reference. After incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody, the antigen-antibody reaction was detected by enhanced chemiluminescence system and Quantity One analysis software (Bio-Rad, San Francisco, California, USA).

2.4. Immunohistochemistry assay

Brain tissues were fixed with 4% paraformaldehyde (sigma, USA) fixed at 4 °C for 24 h. Brain tissues were rinsed with PBS, 30%, 50% and 70% alcohol in turn. Next, brain tissues were embedded in paraffin and dehydrated. Tissue sections (4 μm) were cut. The paraffin samples were removed from the sections with xylene and rehydrated with graded alcohol gradually. The slides were treated with 1% triton-100 for 15 min and then incubated with 3% H_2O_2 to block endogenous peroxidase activity. After blocking with 5% goat serum, the slides were incubated successively with primary antibody and secondary antibody. At last, the slides were performed by staining and observed through a Nikon microscope (Image Systems, Columbia, MD).

2.5. Enzyme-linked immunosorbent assay (ELISA)

For ELISA analysis, blood was obtained at the end of experiment and centrifuged at 3000 rpm for 10 min to collect serum. The serum levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), nitric Oxide (NO), interleukin (IL)-6, IL-10 and tumor necrosis factor alpha (TNF-α) were determined by commercially available ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol.

2.6. Immunofluorescence

After blocking with 5% goat serum, the slides were incubated LC3 antibody at 4 °C overnight. Slides were incubated with fluorophore-conjugated secondary antibody for 1 h at room temperature. After washing with PBS, slides were counterstained with DAPI for 10 min. A fluorescence microscope was used for LC3 co-localization analysis.

2.7. Statistical analysis

All statistical analyses were performed using SPSS 16.0 software. The statistical significance of the study was analyzed using Student's t test. Comparing two groups, the difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Vitexin reduced brain infarction in MACO model rats

TTC staining was used to estimate the severity of cerebral infarction. In the stained sections, normal tissue presented deep red while the infarct area was white. As shown in Fig. 1B, small brain infarction was observed in the healthy control group and sham group. Compared with sham group, the brain infarction volume in MCAO group was remarkably increased ($P < 0.01$). However, treatment with vitexin