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Autophagy induction by hispidulin provides protection against sevoflurane-induced neuronal apoptosis in aged rats

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ABSTRACT

Aim: Sevoflurane is a general anesthetic, which has been found to cause cognitive and memory deficit in elderly. This study is designed to explore the neuroprotective effect of hispidulin, a natural flavone compound, against sevoflurane-induced cognitive dysfunction in aged rats.

Materials and methods: Human neuroglioma cell line H4 was used as cellular model in our study. The apoptosis of H4 cells was determined by DNA fragmentation and flow cytometry. The autophagy of H4 cells was determined by observing GFP-LC3 II puncta and flow cytometry. The levels of marker proteins for apoptosis and autophagy were determined by western blot. The neuroprotective effect of hispidulin was also examined in aged rat model. The impairment of cognitive function by sevoflurane exposure was evaluated by Morris water maze. The apoptotic cell death in hippocampus was measured by TUNEL assay.

Results: Our results showed that hispidulin significantly induced autophagy in H4 cells, which contributed to its protective activity against sevoflurane-induced apoptosis. In addition, our results showed that hispidulin triggered autophagy in AMPK-dependent way. Moreover, the neuroprotective effect hispidulin was verified in aged rat model, which showed that pretreatment with hispidulin significantly attenuated sevoflurane-induced cognitive dysfunction. Meanwhile, our findings revealed that the neuroprotectionin rat model by hispidulin was associated with activation of autophagy and AMPK signaling pathway.

Conclusion: The findings in this present study highlight that hispidulin offers neuroprotection against sevoflurane-induced cognitive dysfunction, which is mediated by autophagy induction through activating AMPK signaling. The present study provides novel information about the underlying mechanism for the neuroprotective activity of hispidulin.

1. Introduction

Elderly patients who undergo major surgery and general anesthesia often present with cognitive dysfunction, lack of concentration and impairment in ability to process information, which are referred to as postoperative delirium and postoperative cognitive dysfunction (POCD) [1]. Although the factors that contribute to POCD have not been fully understood, a number of studies have established the association between use of general anesthetics such as sevoflurane and the onset and progression of Alzheimer's in elderly patients. Study with animal model also showed that sevoflurane anesthesia resulted in decrease of neurogenesis and neuronal cell death in the hippocampus, leading to cognitive dysfunction in aged rats [2]. Mechanistically, sevoflurane exposure in aged rats led to endoplasmic reticulum stress-induced apoptosis of the neurons, which resulted in learning and memory deficits [3]. Therefore, antagonizing against sevoflurane-induced neuronal apoptosis might provide a potentially effective strategy to protect against POCD caused by sevoflurane exposure.

Autophagy, featured by formation of a double-membrane vesicle containing misfolded or aggregated proteins, lipids and organelles, is a defense against apoptotic signal [4]. As to the effect of autophagy on central nervous system, mounting evidence has demonstrated the protective role of autophagy in neurodegeneration [5]. Butterfield et al have also suggested that insufficient autophagy may correlate with cognitive decline and neurodegeneration in the aging process [6]. Moreover, autophagy deficiency has been proposed to contribute to sevoflurane-induced cognitive impairment in elderly [7]. However, whether agents that can induce autophagy could protect neuronal apoptosis and hence alleviate cognitive dysfunction remains elusive.

Accumulating evidence from clinical setting has linked the intake of flavonoids with beneficial effect on brain function, especially in elderly populations [8,9]. Hispidulin is a flavonoid extracted from different

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plant materials such as *SaussureainvolucrataKar. et Kir.*, and several Artemisia and Salvia species [10]. Interestingly, hispidulin has been found to be able to cross blood brain barrier and exert anticonvulsant effect [11]. *In vitro* studies have also showed that hispidulin can activate human benzodiazepine (BZD) receptor as a potent ligand [12]. Furthermore, the neuroprotective effect of hispidulin against neurotoxicity induced by bupivacaine has also been documented in *in vitro* studies [13]. However, the role of hispidulin in sevoflurane-induced cognitive dysfunction and the related underlying mechanisms have never been explored.

2. Materials and methods

2.1. Cell culture

Human neuroglioma H4 cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin (Thermo Fisher Scientific, Shanghai, China), 2mM glutamine (Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) in a humidified incubator at 37 °C with 5% CO_2. Sevoflurane were given in the atmosphere at a concentration of 4.1% using an anesthesia machine. Hispidulin pretreatment in the presence or absence of 3-MA was continued for 6 h before sevoflurane-exposure.

2.2. DNA fragment detection

A Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN) was used to determine the apoptotic population following the manufacture's protocol. Briefly, the cells were lysed with lysis buffer and subjected to centrifugation. The supernatant was added to a streptavidin in-coated microplate and incubated with biotin-labeled anti-histone antibody and peroxidase-conjugated anti-DNA antibody for2 h at room temperature. After washing, absorbance of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was added to the microplate and incubation buffer wash each well for 3 times, and absorbance was read to evaluatethe apoptosis of cells.

2.3. Flow cytometry assay for apoptosis

Apoptotic cell death was measured using an Apoptosis kit (Beyotime, Shanghai, China) following manufacturer's protocol. Briefly, the cells were harvested at a density of 5×10^5 cells/ml and incubated with Annexin V-FITC and propidium in the dark for 15 min before detection using a flow cytometer (Merck Millipore, Germany).

2.4. Quantification of autophagic cells by flow cytometry

Acridine orange (AO) staining was performed to measure the autophagic population as previous described [14]. Following treatment, H4 cells were stained with AO (1μg/ml) for 15 min before the florescence signal was detected by a flow cytometer (Merck Millipore, Germany). Autophagic cell population was quantified according to the intensity of red fluorescence at 630 nm.

2.5. Fluorescence assay for autophagic flux

H4 cells were transfected with mRFP-GFP-LC3 expressing plasmid following manufacturer's instructions (Roche, Shanghai, China). Analysis of fluorescence was performed using an Olympus (Tokyo, Japan) and imaging software.

2.6. Western blot assay

The proteins were extracted from hippocampus and cells using a commercial lysis buffer as previously described (Beyotime, Shanghai, China). The protein content was determined by a BCA assay kit (Thermo Scientific, Shanghai, China). Western blotting was performed following standard protocols with 50μg protein was loaded for each sample. Proteins were detected with specific primary antibodies against cleaved caspase-3, Bcl-2, and Bax from Abcam (Shanghai, China), primary antibodies against p-AMPK, AMPK, p-mTOR and mTOR from Santa Cruz (Santa Cruz, CA), primary antibodies against LC3 I and LC3 II and p62 from Cell Signaling (Danvers, MA), and primary antibodies against β-actin from Beyotime (Shanghai, China). The immunoblots were detected with HRP-conjugated secondary antibody form Beyotime (Shanghai, China). An ECL system was used to perform the western blot analysis (Vector Laboratories, Burlingame, CA).

2.7. Knockdown of AMPKαwith siRNA

The siRNA oligos for AMPKα gene knockdown were purchased from Genepharma (Shanghai, China). H4 cells in logarithmic growth phase were plated in 12-well plates at a density of 3×10^5 cells per well and transfection was conducted using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.8. Animals and treatment

Male Sprague-Dawley rats (20 months age) were purchased from Shandong University Laboratory Animal Center. The experimental rats were kept under SPF conditions with 12 h:12 h light dark cycle and air conditioning set for 23 °C for 1 weeks before the entry of experiment for accommodation. Food and water was given ad libitum. The experimental protocol was approved by the Medical Ethics Committee of Ji'nan Central Hospital Affiliated to Shandong University. A total of 32 rats were allocated into four experimental groups as the following: Vehicle (V) group, sevoflurane (Sevo) group, hispidulin (His,Solarbio Science & Technology Co., Ltd., Beijing, China.) group and sevoflurane + hispidulin (Sevo + His) group. The rats in His and Sevo + His group received sevoflurane in the environment containing 4% sevoflurane for 4 h. Hispidulin was given *via* intraperitoneal injection as a single dose at a dosage of 40mg/kg at12 h before sevoflurane exposure. Chloroquine was given in combination with hispidulin at a dosage of 20 mg/mg.

2.9. Morris water maze

Morris water maze test was performed to evaluate the learning and memory function as previously described [15]. Acquisition test were conducted in successive four days. Two training sessions were performed on each with a 15 min' break between them. In the training session, every rat was put in front of the tank wall, at a designated starting point in different quadrants of the maze every day. Same starting point was employed in the training session for all animals. When rat swam for aperiod of time before climbing onto the platform and remained there for more than 3 s, the swimming time was recorded and defined to be the latency for seeking escape. In the case that the rat failed to locate the platform within2 min, or it was ushered to the platform and remained there for 30 s, the latency was documented as 120 s. Following the evaluation of latency, spatial probe trial was performed with removal of the platform. Animal model was put in front of the wall at any starting point not neighboring to the platform. The times of the animal model to pass the original site of platform in 2 min was recorded.

2.10. TUNEL assay for apoptotic cell death in hippocampus

TUNEL assay was performed using a TUNEL kit (Beyotime) as previously described [16]. The processed sample was incubated with TUNEL reaction mixture for 1 h at 37 °C in the dark. The TUNEL-