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Moderate hypothermia protects increased neuronal autophagy via activation of extracellular signal-regulated kinase signaling pathway in a rat model of early brain injury in subarachnoid hemorrhage

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ABSTRACT

Moderate hypothermia (MH) used as treatment for neurological diseases has a protective effect; however, its mechanism remains unclear. Neuronal autophagy is a fundamental pathological process of early brain injury in subarachnoid hemorrhage (SAH). We found that moderate activation of autophagy can reduce nerve cells damage. In this study, We found that MH can moderately increase the level of autophagy in nerve cells and improve the neurological function in rats. This type of autophagy activation is dependent on extracellular signal-regulated kinase (ERK) signaling pathways. The level of neuronal autophagy was down-regulated significantly by using U0126, an ERK signaling pathway inhibitor. In summary, these results suggest that MH can moderately activate neuronal autophagy through ERK signaling pathway, reduce nerve cell death, and produce neuroprotective effects.

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1. Introduction

SAH is a type of cerebrovascular disease that is caused by intracranial vascular rupture due to various factors and seriously threatens human health. The cure rate for SAH has increased significantly with the advances in surgical techniques and medical equipment. However, its morbidity and mortality are still high. The surviving patients also have different degrees of cognitive decline, obstacles and dysfunctions; their quality of life is negatively affected; and their communities and families are bestowed a heavy burden [1]. SAH may induce early brain injury (EBI) and delayed brain injury, which can manifest as cerebral edema, intracranial hypertension, cerebral infarction, neurological disorders, and disturbances of consciousness. These symptoms may result from inflammation, cerebral vasospasm, apoptosis, and autophagy [2]. However, the current pathogenesis and treatment strategy for SAH remain poorly understood and thus warrant further study.

Hypothermia at 28–35 °C was defined as moderate hypothermia (MH) [3]. MH has made great breakthroughs in basic and clinical research. MH may exert neuroprotective effects by reducing the markers of apoptotic pathway in TBI rats [4]. MH can reduce the risk of vasospasm and DCI in patients with SAH, improve functional outcomes, and reduce mortality [5]. However, only few studies focused on the effects and molecular mechanisms of MH in SAH.

Autophagy is a key homeostatic process wherein cytosolic proteins and organelles are degraded and recycled. This process maintains cellular homeostasis and survival [6]. As a clearance pathway, autophagy exerts protective effects in multiple neurological disease models [7]. Autophagy can be activated in the SAH model, and this activation has neuroprotective effects [8]. However, the mechanism of autophagy activation is complex and involves multiple signal pathways. The up-regulation of cell surface estrogen receptor alpha is associated with mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) activity and promotes autophagy maturation in vitro [9].

This study aims to investigate the effects of MH in EBI after SAH in rats. In particular, we elucidate whether or not treatment with MH after SAH would protect rats against EBI and would moderately activate neuronal autophagy. Possible underlying mechanism(s) of any actions are also analyzed.

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2. Materials and methods

2.1. Animals

The experimental protocol was approved by the Animal Care and Use Committee of Central North China science and Technology University of China (No. 2013-99) and has conformed to the standards of the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health of the United State of America. All efforts were taken to minimize the number of rats used and their suffering. Adult males Sprague Dawley (SD) rats that weigh 350–450 g and are 10–12 weeks old were supplied by Vital River Laboratories Ltd., Beijing, China. The license number was SCXK (Beijing): 2015-003. The animals were fed and given free access to water during experiments conducted in the Center of North China Science and Technology University and were allowed to adapt to feeding 14 days before the experiment. Feeding environment: temperature 18–26 °C, relative humidity 40–70%, and temperature in the general rat feeding box must be 1–2 °C higher than that in the environment and must have high humidity ~10%. Noise was below 85 dB, and ammonia concentration was below 20 PPm. Light and shade with alternating light were provided every day.

2.2. Experimental design

2.2.1. Experiment I

Rats were randomly divided into SHAM, SAH model, and MH + SAH group to determine the neuroprotective effect of MH treatment. SAH model and MH + SAH group were further divided into four subgroups: 6, 24, 48, and 72 h. The neurological function of rats was detected by Garcia score table, The brain edema index was detected by dry-wet method and HE staining. The morphology of neurons in hippocampus CA1 area was also observed.

2.2.2. Experiment II

Rats were randomly split into three groups: SHAM, SAH model, and MH + SAH group to observe the changes on autophagy-related protein Beclin-1 and LC3 after MH treatment. Beclin-1 and LC3 in the hippocampus of SAH rats were detected 24 h after SAH by using IHC and Western blot.

2.2.3. Experiment III

Rats were randomly divided into four groups: SAH + vehicle (equal amount of DMSO solution), MH + SAH, U0126 + SAH dissolved in DMSO at a dose of 0.05 mg/kg (R & D systems, Minneapolis, MN, USA), and U0126 + MH + SAH (dissolved in DMSO at a dose of 0.05 mg/kg) to evaluate the role of the ERK signaling pathway in activating autophagy induced by MH. The neurological function of rats was detected by Garcia score table and the brain edema index was detected in the hippocampus by dry-wet method. The expression of phosphorylated ERK1/2 (*p*-ERK1/2) Beclin-1 and LC3 were detected in the hippocampus by Western blot.

2.3. Establishing the SAH model

The SAH model was induced by endovascular perforation. While the mouse was under pentobarbital (50 mg/kg) anesthesia, the left carotid artery and its branches were exposed and separated. The left external carotid artery was cut, and a 4-0 monofilament suture was advanced into the internal carotid artery through it until resistance was felt. Then, the suture was inserted further to puncture the vessel and to induce SAH. The sham rats underwent the same procedure but without vessel puncture.

2.4. Manipulation of temperature

Frontal cortex brain temperature was monitored with a digital electronic thermometer (model DP 80; Omega Engineering, Stamford, CT) and a 0.15 mm-diameter temperature probe (model HYP-033-1-T-G-60-SMP-M; Omega Engineering), which was inserted 4.0 mm ventral to the surface of the skull. The probe was removed before FPI and replaced immediately after injury. Rectal temperatures were measured with an electronic thermometer with analog display (model 43 TE; YSI, Yellow Springs, OH) and a temperature probe (series 400; YSI). The 32 °C brain temperature was achieved by immersing the anesthetized rat body in ice-cold water. The skin and fur of all 21 animals were protected from water by placing the animal in a plastic bag (head exposed) before immersion. Animals were removed from the water bath when the brain temperature was reduced to within 2 °C of the target temperature. Approximately 30 min was required to reach the target brain temperatures, which were maintained for 4 h under general anesthesia in room temperature through the intermittent application of ice packs as needed. A brain temperature of 37 °C was achieved under general anesthesia with a heating blanket.

2.5. Neurological score

Neurological score were evaluated by two blinded investigator [10], the animals from each group were tested for their neurological function by a modified Garcia Scale method for spontaneous activity, limb symmetry, forearm extension, climbing response, body sensation, and responsive touch. The scores for each part were added to obtain the total score of neurological function. According to the scale, a low score indicates severe nervous system injury. On the contrary, a high score indicates less nerve damage. The lowest score is 3 and the highest score is 18.

2.6. Water content in brain

The brain was separated into four parts, including the left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was immediately weighed (wet weight) and promptly dried for 72 h at 105 °C (dry weight). The water content was then measured [11].

2.7. HE staining of rats' brains

Rats were subjected to deep anesthesia by 10% chloral hydrate. All rats were perfused transcardially by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brain was subsequently harvested, and the brain tissue spanning from the optic chiasm to the cerebral transverse fissure was resected. The tissue was embedded in paraffin, cut into coronal sections (5 μm), and stained with hematoxylin and eosin. Sections were observed under an optical microscope.

The sections were cut in a microtome and allowed to adhere to glass slides with polylysine. Images of the ipsilateral hippocampus were captured at 400 × by using a microscope (Nikon Labophot; Nikon USA, Melville, NY). Specimens were examined by two pathologists (blinded to group conditions) to identify cell death based on characteristic cellular morphological changes. Eight rats were included in each of the four groups.

2.8. IHC analysis of rats' brains

The immunoreactivity of LC3 and Beclin-1 was detected by subjecting the 4 l m-thick formalin-fixed, OCT-embedded sections to IHC analysis. Endogenous peroxidase was blocked with 3% H_2O_2

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