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Effects of intermedin on autophagy in cerebral ischemia/reperfusion injury

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ABSTRACT

Objective: This study aimed to evaluate the effects of intermedin (IMD) on autophagy in cerebral ischemia/reperfusion (I/R) injury (CIRI).

Methods: Sixty rats were randomly averaged into four groups: sham, ischemia/reperfusion (I/R), IMD, and 3-methyladenine (3-MA). In the sham group, the right common carotid artery, external carotid artery, and internal carotid artery were detached, and no monofilament was inserted. In the other groups, two hours after cerebral ischemia, the rats were injected through the lateral ventricle with normal saline for I/R group, IMD for the IMD group, and 3-MA for the 3-MA group for 24 h. The cerebral injury was assessed by evaluation of neurological function, hematoxylin and eosin (H & E) staining, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The expressions of autophagy associated proteins, such as microtubule-associated protein 1 light chain 3 (LC3), Beclin1, and sequestosome 1 (P62) were analyzed using immunohistochemistry staining and western blot. Meanwhile, transmission electron microscopy was used to investigate the ultrastructure of the brains.

Results: IMD could reduce neuron cell damage and infarction formation and has a protective effect against CIRI as 3-MA. The levels of LC3II/LC3I and Beclin1 were significantly decreased and the P62 level was significantly higher in the IMD group compared with I/R group, which is similar to the effect of 3-MA on CIRI.

Conclusions: IMD has a similar effect as 3-MA, can reduce pathological neuronal injury and protect the brain against CIRI in rats by attenuating the effects of autophagy. Our findings provide evidence for IMD's protective effects in relation to ischemic cerebrovascular diseases.

1. Introduction

Cerebrovascular disease is a common clinical disease that seriously endangers human health and life, and the morbidity and mortality are increasing with aging (Turner et al., 2013). Cerebral ischemia affects many vascular areas, leading to tissue hypoxia, nerve cell degeneration and necrosis, and cell death. Therefore, restoration of the blood supply to an organ deprived of blood flow is necessary and is considered as a rationale for clinical therapy (Schaller and Graf, 2004). Even though reperfusion may improve the outcomes in some clinical trials, it can also paradoxically cause delayed nerve cell injury and tissue damage, which is so-called “cerebral ischemia/reperfusion (I/R) injury (CIRI)”, and exacerbate the brain injury (Wang et al., 2017a). Thus, it is very important to understand the mechanism and pathophysiology of CIRI and to attenuate CIRI for the treatment of cerebrovascular diseases.

Many studies have discussed and shown that autophagy is one of the pathological mechanisms of CIRI. Autophagy is a cell-specific self-digestion process that allows phagocytosis of macromolecules and organelles in the cytoplasm through the lysosomal system during starvation, differentiation, and cell growth to maintain cell survival, differentiation, growth, and stability (Zhao et al., 2017). Macroautophagy is the main pathway used to eradicate dysfunctional components, which involves the formation of autophagosome around the organelles marked for degradation. The formation of autophagosome is regulated by

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several conserved genes from yeast to mammals. Microtubule-associated protein 1 light chain 3 (LC3), the mammalian ortholog of yeast autophagy-related gene 8 (Atg8), is involved in autophagosome formation during autophagy and is a specific protein marker of autophagosome (Cui et al., 2013). Some studies have reported that the expression of Beclin1, the mammalian ortholog of yeast autophagy-related gene 6 (Atg6), is downregulated in breast carcinoma, and the loss of *Beclin1* contributes to an increased rate of cancer in association with reduced autophagy (Anzell et al., 2017), being implicated in the autophagy process. Studies showed that suppressing Beclin1-dependent autophagy attenuated neuronal cell death in cerebral ischemic stroke and initiated hypertrophic growth (Cheng et al., 2017; Guo et al., 2017). Among the other proteins involved in the regulation of autophagy process, autophagy-selective substrate-sequestosome 1 (P62) plays a key role in the formation of cytoplasmic protein inclusion bodies and the level of P62 is increased when autophagy is inhibited (Chen et al., 2012).

Intermedin (IMD) is one of the members of the calcitonin gene-related peptide (CGRP) superfamily, whose precursors and pro-adrenomedullin (AMD) precursors are almost identical in amino acid and nucleotide sequence and have some similarity in function. IMD has a certain protective effect for cardiovascular, cerebral, and renal I/R injury (Morimoto et al., 2007). It can also inhibit pro-inflammatory cytokine release and inhibit oxidative stress (Kovaleva et al., 2016). Studies have shown that IMD can significantly reduce the peripheral blood pressure and the total peripheral resistance of rats, relax blood vessels, and increase local blood perfusion (Schonauer et al., 2017; Wimalawansa, 1997; Zhang et al., 2017; Wu et al., 2017; Qiao et al., 2013). Although it was speculated that IMD may protect against CIRI through some effects on autophagy process (Bell et al., 2007; Kuma et al., 2004), the protection mechanism remains poorly understood.

Here, our study investigated whether IMD protects against CIRI through attenuating autophagy by analyzing the relevant factors and proteins, like LC3, Beclin1, and P62.

2. Materials and methods

2.1. Animals

This study was approved by the Ethics Committee of North China University of Science and Technology, China. And all experiments were performed in accordance with relevant guidelines and regulations.

Male Sprague-Dawley mice (6–7 weeks, weight: 260 ± 10 g) were purchased from the Institute of Hygienic Environmental Medicine, Academy of Military Medical Sciences, China (License: SCXK- (Army) 2009–003). All rats were housed in the Barrier Laboratory of Experimental Animal Center, North China University of Science and Technology. The indoor temperature was 20–25 °C. The rats were fed with standard and free to drinking. It's at least one week for the rats to adapt to the environment. Before model establishment, the rats were fasted for 12 h but with drinking.

Sixty rats were randomly allocated into four groups: sham (n = 15), I/R (n = 15), IMD (n = 15) and 3-methyladenine (3-MA; n = 15) groups. In the sham group, the right common carotid artery, external carotid artery, and internal carotid artery were detached, and no monofilament was inserted. In the other groups, two hours after cerebral ischemia, the rats were injected through the lateral ventricle using a micro-syringe with normal saline (NS, 25 μl each time)for I/R group, IMD (100 mg/kg) for the IMD group, and 3-MA (200 nmol) for the 3-MA group for 24 h (Jiang et al., 2015).

2.2. Establishment of the focal cerebral I/R model of middle cerebral artery occlusion (MCA)

The focal cerebral I/R model was generated via thread embolism of the MCA as previously described (Longa et al., 1989). Rats were kept in supine position at a constant temperature of 38 °C, and isoflurane anesthesia was administered. The right common carotid artery, internal carotid artery, external carotid artery, superior thyroid artery, and vagus nerve were isolated. Following this, the distal and proximal sites of the right side of the external carotid artery and the superior thyroid artery were ligated. Then, the middle of two ligation sites of the right external carotid artery was cut with a neurosurgical microscissors. The distance from the breakpoint to the proximal site should be > 2 mm to avoid slippage. Next, the occipital artery (as far as possible away from the external carotid artery) was ligated and severed. After the arterial clip clamping the proximal common carotid artery and the distal internal carotid artery, the right external carotid artery was raised and the bifurcation of it was cut for a small opening with a neurosurgical microscissors. With pulling the external carotid artery to be almost at the same line of internal carotid artery, a suture was inserted through the incision into the right internal carotid artery until the arterial clip. Then, the internal carotid artery clip was loosened and the suture was fast inserted until it couldn't move. Next, the suture was fixed, the time was recorded and the incision was stitched. After 1.5 h, the suture was pulled out and the reperfusion was maintained for 24 h (Bi et al., 2012).

The neurological deficits of all rats were then assessed and recorded using blind neurological function criteria described previously (Longa et al., 1989), and rats that did not meet the criteria were removed from the study. The score of neurologic function was using the 5-point scale method. The higher the score was, the more serious the behavioral disorder was. The success model scored 1–3 point (the success rate was 90%).

2.3. Preparation of paraffin sections

Six rats were randomly selected from each group. They were administered anesthesia with ether, followed by 0.9% saline rapid heart perfusion, and 4% paraformaldehyde perfusion when there is clear liquid out of the auricle. The rats were then quickly decapitated, and the brain was preserved in 4% paraformaldehyde for 24 h. Three-millimeter sections of coronal brain tissue at 2 mm after the optic chiasm were cut. After routine embedded in paraffin blocks, the coronal brain tissue was cut into 4 μm-thick slices for hematoxylin and eosin (H & E) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and immunohistochemical staining.

2.4. H & E staining

Slices were deparaffinized with xylene and gradient alcohol and immersed in H_2O for 30 s with agitation by hand. The slide was then dipped into a Coplin jar containing Mayer's hematoxylin for 5 min and rinsed with H_2O for 1 min. The slide was stained with 1% eosin Y solution for 2 min with shaking. After that, the sections were dehydrated in 95% alcohol twice and 100% alcohol twice for 30 s each. Next, the alcohol was extracted with two wash with xylene. Finally, the slide was covered with a coverslip, mounted, and observed under a microscope.

2.5. TUNEL staining

Paraffin sections were dewaxed into the water, blocked in 3% H_2O_2 for 10 min, and then incubated with compound digestion solution at 37 °C for 20 min. After that, sections were incubated with dTUNEL mixture at 37 °C for 1 h or 4 °C overnight. Next, peroxidase (POD) conversion solution (anti-fluorescein isothiocyanate [FITC]-HPR) was added and incubated at 37 °C for 40 min. Slices were then stained with diaminobenzine (DAB) and hematoxylin staining, and sealed with neutral gum seal. Positively stained cells were counted from 5 randomly selected views under the microscope at high magnification.