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S-oxiracetam ameliorates ischemic stroke induced neuronal apoptosis through up-regulating α7 nAChR and PI3K / Akt / GSK3β signal pathway in rats

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ABSTRACT

Ischemic stroke, the main reason for severe disabilities in the world, is associated with a high incidence of sensorimotor and cognitive dysfunction. In this study, we use the middle cerebral artery occlusion/reperfusion (MCAO/R) model in rats and oxygen glucose deprivation/reoxygenation (OGD/R) model in fetal rat primary cortical neurons to investigate whether and how S-oxiracetam (S-ORC) protect brain injury from ischemic stroke. The results revealed that S-ORC reduced brain infarct size and lessened neurological dysfunction after stroke. Further study demonstrated that S-ORC diminished TUNEL positive cells, increased cell viability, decreased LDH activity, and inhibited cell apoptotic rate. Furthermore, S-ORC inhibited neuronal apoptosis by activating the PI3K/Akt/GSK3β signaling pathway via α7 nAChR, which was evidenced by α7 nAChR siRNA. In conclusion, our findings strongly suggest that S-ORC could be used as an effective neuroprotective agent for ischemic stroke due to its effect in preventing neuronal apoptosis.

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1. Introduction

Ischemic stroke is a debilitating clinical disorder associated with a high incidence of sensorimotor and cognitive dysfunction, and it has become the second leading cause of death and the most important cause of disability in many countries. However, effective neuroprotective treatments is lacking (Ding et al., 2015a; Harris et al., 2016; Li et al., 2013). MCAO in rodents is considered to be a convenient, reproducible, and reliable model to mimic human ischemic stroke and has been widely used in numerous studies in rats (Carmichael, 2005; Li et al., 2013). Reperfusion after ischemia may aggravate brain injury, and ischemia/reperfusion (I/R) can lead to death of neurons (Sun et al., 2010). Neuronal death may occur by both necrosis and apoptosis, with necrosis tending to occur soon after ischemic insult and apoptosis taking precedence later (Chen et al., 1998). Furthermore, apoptosis is an important factor in causing neuronal death after brain I/R injury (Wang et al., 2014b). Accordingly, agents that can prevent neuronal apoptosis are believed to have therapeutic potentials toward brain ischemic stroke.

Early studies reported that most of the apoptotic cells in the ischemia core were identified as neurons (Li et al., 1995), and that damage of neurons causes severe learning and memory impairment (Jarrard, 1993). Thus, targeting and preventing apoptosis was regarded as a rational therapeutic strategy to limit cerebral infarct volume after stroke (Qi et al., 2016). Seventeen nAChR subunits have been identified in vertebrate species (α1-α10, β1-β4, γ, δ an ε), and most of them, except α8, are expressed in humans and in different mammalian species (Inmaculada et al., 2013; Millar and Gotti, 2009). Accumulating evidences indicate that alpha-7 nicotinic acetylcholine receptor (α7 nAChR) and α4β2 nAChR are involved in the neuroprotection against glutamate, and only α7, but not α4β2 nAChR, is essential for reversing glutamate-induced neuronal death (Cui et al., 2013; Shen et al., 2010). The α7 nAChR, which is involved in pro-survival intracellular pathways (Terrando et al., 2015; Truong et al., 2015), is expressed in the central nervous system (CNS) and is thought to play a role in a wide variety of

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psychiatric and neurological disorders (Kalkman and Feuerbach, 2016). In the cardiovascular system, the α7 nAChR was reported as an important protector in myocardial I/R injury, stroke and hypertension (Li et al., 2014), and the activation of α7 nAChRs plays an important role in neuronal survival (Yu et al., 2011). Furthermore, a number of studies support the involvement of α7 nAChRs in pro-survival cell signaling, engaging the PI3K/Akt signaling pathway (Cui et al., 2013; Huang et al., 2012). Such signaling was shown to protect cultured brain cells from apoptosis (Parada et al., 2010). Moreover, increased activity of GSK3β, a downstream kinase of Akt, has been implicated in neuronal death, as previous studies proved that the PI3K/Akt/GSK3β signaling pathway inhibited cell apoptosis or death induced by hypoxia and I/R (Arslan et al., 2013; Li et al., 2012; Wang et al., 2016; Zhang et al., 2014).

S-oxiracetam (S-ORC), also known as (S)-2-(4-hydroxy-2-oxopyrrolidin-1-yl) acetamide, is the S enantiomer of oxiracetam (ORC), which is a nootropic drug known for its effect in improving cognition and memory, and the anti-neurodegenerative effect on ischemic stroke (Hlinak and Krejci, 2005; Wang et al., 2014a). Our previous studies showed that S-ORC alleviates BBB dysfunction by regulating tight junction proteins (TJPs) and upregulating P-glycoprotein function, and subsequently protects against ischemic stroke (Huang et al., 2017). In this study, we investigated the neuroprotective role of S-ORC treatment against rat MCAO model of ischemic stroke, and further elucidated the possible molecular mechanism of S-ORC by performing the OGD/R model on fetal rat cortical neurons, in order to provide information for future studies of the agents that could prevent neuronal death caused by ischemia/reperfusion.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–280 g body weight) were purchased from Zhejiang Laboratory Animals Center (Zhejiang Academy of Medical Science, Hangzhou, China). All animals were cared for in the Animal Center of China Pharmaceutical University, under standard conditions (12 h light/dark cycle; 23 ± 2 °C; 55 ± 10% humidity) with free access to food, water and standard laboratory chow. E15-18 embryos of pregnant Spraguee-Dawley rats were purchased from Qinglongshan Animal Farm of Nanjing, China. All studies were in compliance with institutional guidelines of China Pharmaceutical University (Nanjing, China).

2.2. Implementation of MCAO/R model

Male Sprague-Dawley rats with similar degrees of body weight were randomly divided into 6 groups: the sham (saline), MCAO/R (saline), S-ORC (0.48 g/kg, 0.24 g/kg or 0.12 g/kg) and ORC (0.24 g/kg) groups. Rats were fasted but with water supply the night before surgery. Rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and MCAO surgery was performed according to Longa et al. with a few modifications (Longa et al., 1989). After 1.5 h of MCAO, reperfusion was accomplished by withdrawing the filament. The rats of the sham group received all the surgical procedures without the filament inserted. At 2.5 h after MCAO, agents were administrated intravenously for seven days, with a volume of 0.5 ml/100 g/day.

2.3. Neurological deficit

At seven days after MCAO, neurological deficit scores were assessed according to the method of (Longa et al., 1989). Five categories of motor neurological functions were scored: 0: no deficits, 1: contralateral forelimb flexion when tail is lifted, 2: decreased resistance to lateral push on a smooth surface, 3: spontaneous circling movement in the contralateral direction, 4: no spontaneous movement.

2.4. Assessment of infarct size

Seven days following MCAO, rats were sacrificed under deep anesthesia to obtain the brain sample. Brain infarct area was evaluated by using 2, 3, 5-triphenyltetrazolium chloride (TTC) (Lingjin Co. Ltd, Shanghai, China) staining method as previously published (Lv et al., 2011). 5 pieces of (2-mm thick) coronal sections were collected from the brain. The brain sections were stained in 2% TTC at 37 °C for 15 min in the dark and photographed. Viable brain tissue areas turned into dark red because of the intact mitochondrial function and the infarct areas remain unstained. The infarct tissue areas were measured by Image-Pro Plus software and obtained the final results using following formula: The infarct area = (total infarct area/whole brain section area) × 100%.

2.5. Determination of the concentration of GSH-PX and acetylcholinesterase (AChE) activity in the rat brain

After animals were sacrificed, brain tissues were collected and prepared as homogenate and the determination of the concentration of GSH-PX and AChE activity in the rat brain were performed. Experiments were conducted using GSH-PX assay kit (Jiancheng, Nanjing, China) and AChE assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instruction by a person who was blinded to the group assignment of the experimental animals.

2.6. Immunofluorescence and TUNEL staining

Three days following MCAO, the detection of apoptotic neuron cells after MCAO was performed using immunofluorescence and TUNEL Staining according to the method of (Xu et al., 2015). Rats of each group were anesthetized and under-went perfusion with normal saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffered saline (PBS) to fix the tissue. Brains were removed and post-fixed in 4% paraformaldehyde for 24 h. Coronal slides (30 μm) of freshly frozen brain were cut using a cryotome followed by immunofluorescence and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The slides were washed three times in TBS (50 mM Tris-HCl, pH 7.4; 150 mM NaCl) plus 0.3% Triton X-100 with gentle agitation and block in 10% normal serum in TBS for 2 h at room temperature. Then, drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper and further incubated with anti-NeuN antibody (1:200, OriGene Technologies, Inc. Rockville, USA.) overnight at 4 °C. Subsequently, the slides were incubated with Goat Anti-Mouse IgG/Cy3 (1:200, Bioss, Beijing, China) for 2 h at room temperature after rinsing three times with TBS. The rinsed slides were stained using TUNEL kit (Keygenbiotech, Nanjing, China) according to the manufacturer's instruction by a person who was blinded to the group assignment of the experimental animals. After rinsing three times with TBS, the slids were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Bioss, Beijing, China) for 10 min prior to capturing images by using Carl Zeiss fluorescence microscope, and images were taken with a CCD Spot camera equipped with image acquisition software (Axio Vision; Carl Zeiss). Green TUNEL dots located in red neurons with a blue nucleus were identified as apoptotic neurons. The total number of neurons and apoptotic neurons of sections from each rat were counted and then ratios were calculated.

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