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Clonidine preconditioning alleviated focal cerebral ischemic insult in rats via up-regulating p-NMDAR1 and down-regulating NMDAR2A / p-NMDAR2B

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ABSTRACT

A brain ischemia rat model was established by middle cerebral artery occlusion (MCAO) for 2 h and reperfusion for 4 h to investigate the underlying mechanism of the neuroprotection action of clonidine, a classical alpha-2 adrenergic agonist, on cerebral ischemia. Clonidine and yohimbine were intraperitoneally given to the rats each day for a week before ischemia. Neurological deficits evaluations were carried out at 6 h after operation. TTC staining method was used to measure the volume of brain infarction. Expression levels of NMDAR1, NMDAR2A, NMDAR2B were assayed by western blotting. Our data demonstrated that clonidine pretreatment significantly improved the neurological deficit scores and reduced the brain infarct volumes of the rats. Furthermore, protein expression level of p-NMDAR2B in cortex was significantly up-regulated whereas that of p-NMDAR1 was decreased when compared with the sham-operated rats. Remarkably, clonidine treatment led to significant down-regulation of p-NMDAR2B and NMDAR2A in addition to enhancement of the expression level of p-NMDAR1 in cortex. This is the first report illustrating the neuroprotective role of clonidine may be mediated through modulation of the expression levels of p-NMDAR2B, NMDAR2A and p-NMDAR1 during cerebral ischemia.

1. Introduction

Brain ischemia, the second predominant reason of human expiration and long-range crippledom worldwide, remains a very intractable clinical issue (Moskowitz et al., 2010; Hu et al., 2013, 2015; Lai et al., 2014, 2011). Until now there is less efficacious therapy for treatment of brain ischemia. Since the early 1970s, alpha 2-adrenoceptor agonists have been used as anti-hypertension agents in patients with hypertension and to lower afterload in aortic operation and congestive heart failure successfully. Alpha 2-adrenoceptor agonists also produce various responses, including sedation, analgesia, antianxiety, et al. (Zhang and Kimelberg, 2005; Eisenach et al., 1996; Kamibayashi and Maze, 2000). Lately, many reports show that alpha-2 adrenergic receptor agonists ameliorate the neurological scores, improve the histomorphological outcome after brain ischemia when injected during and before ischemia, indicating that adrenaline release could also be involved in post-ischemic damage (Zhang and Kimelberg, 2005; Maier et al., 1993; Maiese et al., 1992; Hoffman et al., 1991a, 1991b; Cosar et al., 2009; Sato et al., 2010; Goyagi et al., 2009). The potential mechanisms underlying these neuroprotective effects comprise: (1) reduction of the immoderate release of norepinephrine and glutamate during energy unbalance (Zhang and Kimelberg, 2005; Boehm, 1999; Scanziani et al.,1993; Talke and Bickler, 1996); (2) depression of voltage-operated calcium channels, which are triggered by NMDA- evoked depolarization and be involved in the neuron calcium overload (Nacif-Coelho et al., 1994; Surprenant et al., 1992); (3) activation of G protein-linked inward rectifying K^+ channels, with secondum cell membrane hyperpolarization (Donello et al., 2001); and (4) inhibition

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of adenylate and guanylate cyclase (Goldstein, 1998; Hein and Kobilka,1995). At the same time, there exist negative reports which arose more interests on the neuroprotective effects of alpha 2-adrenoceptor agonists on brain ischemia (Brede et al., 2011).

In the past several decades, excitotoxicity, a type of neurotoxicity mediated by glutamate, has been at the center stage of stroke research. Among the ionotropic and metabotropic glutamate receptors in the adult CNS, the N-methyl-D-aspartate (NMDA) type of glutamate receptor acts as a hub, by detecting and processing extracellular glutamate signals into diverse intracellular signaling outputs. With the emergence of cellular and molecular biology, scientists are unraveling the mechanisms by which glutamate-mediated activation of the NMDA receptor in health and disease at both the microscopic neuron level and the macroscopic behavior level. Thus, NMDA receptor play a key role in excitotoxicity and in ischemic neuronal death (Gabryel et al., 2012; Ouyang et al., 2014).

The aim of this experiment is to assess the neuroprotective effects of clonidine, an alpha 2-adrenergic receptor agonist, preconditioning on focal cerebral ischemia established by MCAO and explore the underlying mechanisms via NMDA receptor pathway. Clonidine was given to rats every day for a week before the ischemia. The neurological deficit scores were measured and the infarct sizes were determined 6 h after the operation.

2. Materials and methods

2.1. Reagents

Clonidine, yohimbine, 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma (Saint Louis, U.S.A.). Rabbit anti-mouse NMDAR1, NMDAR2B monoclonal IgG were from Cell Signaling Technology (Boston, U.S.A.). NMDAR2A, p-NMDAR2A, p-NMDAR2B polyclonal IgG were from Cell Signaling Technology (Boston, USA). p-NMDAR1 polyclonal IgG were from Millipore (Boston, U.S.A.). GAPDH polyclonal IgG and goat anti-rabbit IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, C.A. U.S.A.). Other reagents used were of analytical grade, which are bought from Shanghai chemical engineering company (Shanghai, China).

2.2. Animals

Adult male Sprague-Dawley rats (NO.00020074) weighing 260–350 g were obtained from the experimental animal center, China Three Gorges University (Yichang, China). All animals were housed in groups of five or six with continuous access to food and water ad libitum and were maintained on a 12 h light/dark cycle regulated at 23 °C room temperature. The experiments began at least 7 days after their arrival. Handling and experimental procedures on all animals were in accordance with the institutional and National Research Council's guideline for animal experiments, which complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.3. Surgery

Focal cerebral ischemia model was made using the intraluminal filament technique. Chloral hydrate (350 mg/kg, i.p.) was administered as anesthesia; it was maintained throughout the operation. All other procedures were carried out as Zuo described (Zuo et al., 2012).

2.4. Experimental treatment

Rats were randomly divided into the sham-operated group (n=6), the cerebral ischemia model group(n=6), the clonidine (40 μg/kg/d) group(n=6), the yohimbine (5 mg/kg/d) + clonidine (40 μg/kg/d) group (n=6) and the yohimbine (5 mg/kg/d) group (n=6). Clonidine and yohimbine were intraperitoneally given every day for a week before the ischemia. The sham-operated control group and the cerebral ischemia model group were treated with saline for 1 week before induction of sham-operated ischemia and ischemia.

2.5. Evaluation of the neurological deficit scores

Neurological deficits evaluations were carried out at 6 h after MACO by an observer masked to the identity of experimental groups using the following criteria as described by Longa et al. (1989), Zuo et al. (2012): 0, no neurologic deficit or normal function; 1, failure to extend right forepaw fully; 2, circling to right; 3, vert to right; 4, coma or absence of spontaneous motor activity; 5, death. Hence, the higher the score the poor the neurological function is.

2.6. Determination of cerebral infarct

Animals were reanesthetized with chloral hydrate (350 mg/kg) and decapitated after 6 h of modeling. The brains were carefully removed and then sectioned coronally in 2 mm thick sections from 2 mm caudal to the frontal tip with a tissue slicer. The slices were immediately stained with TTC at 37 °C for 10 min and were fixed in 4% phosphate-buffered formalin solution (Zuo et al., 2012). The infarct area in five coronal sections of each brain was determined by scanning the image with a CCD camera (Samsung, Korea) and quantified using specially designed software (Shineso MIC3, China). Accuracy in the measurement of infarct volume was compounded by post-ischemic brain edema that may increase brain volume in the infarcted region. We calculated infarct volumes of each brain by summation of the infarcted area of all brain slices multiplied by the average slice thickness, i.e. [area of infarct in square millimeters×thickness (2 mm)] from the same hemisphere. Result of five coronal sections of each brain of three rats / group was used for the study (Dohar et al., 2008; Yoshimoto and Siesjö, 1999).

2.7. Western blot analysis

To confirm the changes of NMDA receptor subtypes protein levels in the cortex after ischemia, at designated times (6 h after the surgery), animals (n=6) in all groups were killed and used for the Western blot study. All other procedure were carried out as we described previously (He et al., 2008). The following primary antibodies were used: Rabbit anti-mouse NMDAR1, NMDAR2B monoclonal IgG, Rabbit anti-mouse p-NMDAR1, NMDAR2A, p-NMDAR2A, p-NMDAR2B polyclonal IgG. The primary antibodies were diluted (1:1000) in 5% w/v BSA, 1×TBS, 0.1% Tween-20 at 4 °C with gentle shaking, overnight. The results of the Western blot study were scanned and a ROD value was obtained using Scion Image software (Scion Corp., USA).

2.8. Statistical analysis

All results were presented as mean ± S.D. Group differences of neurological deficit scores were assessed by Mann-Whitney *U*-test. Data collected from infarct area was analyzed using one-way ANOVA followed by Duncan's test. Western blot results were analyzed by one-way analysis of variance (ANOVA) followed by the Fisher LSD test (Least-significant difference). Statistical analysis was performed using SPSS 12.0 for windows. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of clonidine on neurological deficit scores

Neurologic examinations were performed at 6 h after MCAO. Ischemia group demonstrated significant increased neurological deficits compared to the sham-operated group ($P < 0.01$). Clonidine 40 μg/kg/d group had lower neurological deficits scores than the ischemia