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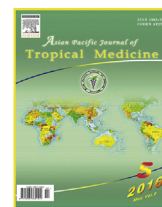

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Mechanism of low molecular weight GTP binding protein RAC1 in injury of neural function of rats with cerebral ischemia reperfusion

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ABSTRACT

Objective: To discuss the mechanism of low molecular weight GTP binding protein RAC1 in the injury of neural function based on building the rat model of cerebral ischemia reperfusion.

Methods: Middle cerebral artery of rats was ligated and the ligature was released to restore the perfusion after 2 h, the rat model of cerebral ischemia reperfusion injury was built, while the middle cerebral artery was ligated. The rats were randomly divided into the sham group, cerebral ischemia reperfusion group (I/R group) and the group with the injection of RAC1 activity inhibitor NSC23766 (NSC group). The survival and neurological severity score of rats in each group were observed and recorded. Nissl staining was employed to observe the nerve cells, and Western blot to detect expression of RAC1, superoxide dismutase and malondialdehyde.

Results: Number of nerve cells for rats in NSC group was significantly more than that in I/R group, but significantly less than that in sham group, with the statistical difference ($P < 0.05$). The brain water content for rats in NSC group was significantly lower than that in I/R group, but significantly higher than that in sham group, with the statistical difference ($P < 0.05$). The expression of RAC1 and malondialdehyde for rats in NSC group was significantly lower than that in I/R group, but higher than that in sham group; while the expression of superoxide dismutase was lower than that in sham group, but higher than that in I/R group, with the statistical difference ($P < 0.05$).

Conclusions: The inhibition of RAC1 activity can reduce the oxidative stress, reduce the neurologic impairment because of cerebral ischemia reperfusion and thus protect the neural function.

1. Introduction

The ischemia reperfusion injury referred to the situation that in case of ischemia of tissues and organs because of different factors to cause the disorder of cellular metabolism and damage of tissues, when the blood supply was restored, the tissue and cellular injury were aggravated [1–4]. The thrombolysis is a common treatment for the cranial vascular disease. The resulted local cerebral ischemia would lead to the cerebral ischemia reperfusion injury after the thrombolysis and then cause the cerebral edema, cerebral hemorrhage and even death [5]. The nerve cells are extremely sensitive to the ischemic injury that easily causes the death of cells. Because of the difficulty in the renewal of nerve cells, how to guarantee the function of nerve cells after the ischemia reperfusion injury became the focus of clinical researches [6,7]. The related reactions of oxygen free radicals play a key role in the cerebral ischemia reperfusion injury. The activated low molecular weight GTP binding protein RAC1 can produce the reactive oxygen species, while RAC1 plays the role of biological regulation in the pathway that produces the reactive oxygen. The related researches also reported that when the activity of RAC1 was inhibited, the neurologic impairment caused by the cerebral ischemia reperfusion would be reduced, but there had been limited studies on its specific mechanism

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[8–11]. In this study, by building the rat model of cerebral ischemia reperfusion, it was to discuss the mechanism of RAC1 in the neurologic impairment that was caused by the cerebral ischemia reperfusion.

2. Materials and methods

2.1. Laboratory animals

A total of 30 male healthy male Sprague-Dawley rats with the weight of (200–240) g were provided by School of Medicine, Shandong University. They were fed in the feeding room with the reasonable and controllable room temperature, light and humidity freely for one week.

2.2. Instruments and reagents

The automatic biochemical analyzer was purchased from Beckman Coulter (AU680), the biological and functional experimental system from Chengdu Taimeng (BL-420F), the high speed micro centrifuge from Shanghai Sangon Biotech (G508009), the precision single-channel adjustable pipette from Shanghai Sangon Biotech (10–1 000) μL, the gel imaging analysis system from UVP (GDS8000), the electronic balance from Sartorius, the ultra low temperature freezer from Haier (−86 °C); and the biochemical kit from Beckman Coulter, RAC1 primary antibody from Abcam, the goat anti-rabbit secondary antibody from Abcam, superoxide dismutase (SOD) and malondialdehyde (MDA) kits from Maixin Biotech, Western blot kit from Maixin Biotech, citrate buffer antigen retrieval solution from Maixin Biotech and DAB color development kit from Maixin Biotech.

2.3. Experimental methods

2.3.1. Grouping

Rats were randomly divided into three groups, with 10 rats in each group, namely sham group; I/R group (ischemia reperfusion injury group) and NSC group (rats in such group were given the intraperitoneal injection of 2.5 mg/kg NSC23766 solution 6 h after the modeling operation). Rats in sham group and I/R group were given the injection of normal saline at the equal dose after modeling.

2.3.2. Building of R/I model

The middle cerebral artery occlusion [12] was employed to build the rat model of cerebral ischemia reperfusion. Rats were given the intraperitoneal injection of 1% sodium pentobarbital for the anesthesia, with the dose of 50 mg/kg. After the full anesthesia, rats were fixed on the operating table at the supine position. The neck hair was removed and the conventional disinfection was performed. The incision was taken in the middle of neck to fully expose the right common carotid artery, external carotid artery and internal carotid artery. The external carotid artery was ligated. The 0.26 mm heparin nylon suture was inserted in the internal carotid artery through the common carotid artery for (18–20) mm for the ligation to block the blood flow of middle cerebral artery. Two hours after the cerebral ischemia, the ligature was released to realize the reperfusion of blood flow. Forty-eight hours later, rats were executed for the further experiments.

2.3.3. Neurological severity score

The neurological severity score was referred to the recommended modified neurological severity score (NSS) [13], including the evaluation on movement (tail-carrying test, 0–3 points; walking test, 0–3 points), feeling (placing test, 0–1 point; proprioceptive sense, 0–2 points), balancing (balance beam test, stable posture-direct falling, 0–6 points) and reflex (wing, conea, terror and muscle reflex, 0–4 points), with the total score of 18 points. 0 point: normal; 1–6 points: mild impairment; 7–12 points: moderate impairment; 13–18 points: severe impairment.

2.3.4. Nissl staining

The brain tissue was collected from the rat after cutting its head and then embedded with paraffin. The sections of tissue were performed from the optic chiasma and the coronary 4 mm behind, with the thickness of 4 um and Nissl staining. The Nissl body appeared to be purple blue under the light microscope. If the neuron was shrunk, the cytoplasm had the acid staining and Nissl quality. The shrunk or broken nucleus indicated the ischemic neuron.

2.3.5. Brain water content

Three rats were decapitated in each group. The collected brain tissue was weighted to record the wet weight. Afterward, the brain tissue was dried for 24 h and it was weighted again to record the dry weight. The brain water content was calculated according to the equation: brain water content = (wet weight−-dry weight)/wet weight × 100%.

2.3.6. Detection of RAC1, SOD and MDA

The ischemic cardiac muscle tissue of rats was collected to be washed with PBS and then cut into pieces. After being fully lysed, it was centrifuged for 5 min to separate the supernatant. The determination of content was performed for the collected histones. After 2 h of electrophoresis, the transfer was performed. Afterward, the membrane was immersed in Tween20/TBS buffer to be oscillated in the blocking buffer. After adding the primary antibody and secondary antibody respectively, it was washed and then colored with DAB. The gel imaging analysis system was employed for the scanning to calculate the net optical density of research object. The detection of SOD and MDA was performed according to the instruction manual of kits.

2.4. Statistical analysis

The research data was treated with SPSS 20.0. The measurement data was expressed by means ± SD. The one-way ANOVA analysis was employed for the comparison among groups and t test for the comparison between groups. $P < 0.05$ indicated the statistical difference.

3. Results

3.1. Survival of rats

Rats in I/R group had the decreased degree of balance, decreased capacity to act, seizure and convulsion and late eating and drinking after the operation and before the experiment. Two rats died 12 h and 48 h after the operation, with the survival rate of 80.00%. Rats in NSC group had the better recovery of movement and mental status than that in I/R group during the

observation after the operation, with the fast recovery of movement, sometimes seizure and convulsion and basically normal eating and drinking. A rat died 24 h after the operation, with the survival rate of 90.00%. Rats in sham group recovered to be normal after the operation, with the normal eating and drinking and the survival rate of 100.00%. There was no significant difference in the survival rate of rats among three groups ($P > 0.05$).

3.2. Nissl staining of nerve cells

The nerve cells of rats in sham group had the regular morphology and orderly arrangement; the nerve cells of rats in I/R group had the irregular morphology, obvious missing of Nissl bodies and disorderly arrangement, which indicated the abnormal morphology of nerve cells; the nerve cells of rats in NSC group had limited change in the morphology, with the small missing of Nissl bodies and a bit disorderly arrangement. The number of nerve cells for rats in NSC group (124.46 ± 37.05) was significantly more than that in I/R group (59.24 ± 22.17), but significantly less than that in sham group (59.24 ± 22.17), with the statistical difference ($P < 0.05$).

3.3. Neurological severity score

According to the modified NSS, the neural function of rats in sham group was best, while the neural function of rats in I/R group and NSC group had the impairment to the different extent. Rats in I/R group had the most severe impairment and obviously abnormal movement. The statistical analysis indicated that the score of rats in NSC group was significantly higher than that in sham group, but significantly lower than that in I/R group, with the statistical difference ($P < 0.05$), as shown in Table 1.

3.4. Brain water content

Forty-eight hours after the operation, the water content of brain tissue of rats in I/R group and NSC group was increased to the different extent after cerebral ischemia reperfusion injury, which was significantly higher than that in sham group, with the statistical difference ($P < 0.05$). The brain water content for rats in NSC group was significantly lower than that in I/R group, with the statistical difference ($P < 0.05$), as shown in Table 1.

3.5. Expression of RAC1

Western blot was employed to detect the expression of RAC1, while the gel imaging system to measure the density of each group. The expression of RAC1 for rats in NSC group 124.04% ± 33.35% was highest, while the expression of RAC1 for rats in sham group 50.36% ± 14.38% was lowest, which indicated that the cerebral ischemia reperfusion injury was related to the high expression of RAC1. The expression of RAC1 for rats in NSC group was 77.22% ± 20.95%, which was significantly lower than that in I/R group, but higher than that in sham group. It indicated that RAC1 activity inhibitor could inhibit the expression of RAC1 in rats with cerebral ischemia reperfusion injury, with the statistical difference ($P < 0.05$).

Table 1

Neurological severity score, brain water content, and expression of SOD and MDA for rats in each group.

Group	NSS (point)	Water content (%)	SOD (U/mg)	MDA (nmol/mg)
Sham group	0.66 ± 0.09	65.19 ± 3.35	133.52 ± 42.84	3.77 ± 1.02
I/R group	3.07 ± 0.45*	77.53 ± 4.82*	53.23 ± 24.35*	14.47 ± 3.36*
NSC group	2.15 ± 0.39**	70.73 ± 4.36**	92.82 ± 30.89**	7.51 ± 2.12**

Note: Compared with sham group, $^{*}P < 0.05$; compared with I/R group, $^{**}P < 0.05$.

3.6. Expression of SOD and MDA

The oxidative stress indicators of SOD and MDA were measured for rats in each group. The expression of SOD in NSC group was lower than that in Sham group, but higher than that in I/R group; while the expression of MDA in NSC group was significantly higher than that in Sham group, but lower than that in I/R group, with the statistical difference ($P < 0.05$), as shown in Table 1.

4. Discussion

The cerebral ischemia refers to the pathological state caused by the cerebral blood deficiency, while the reperfusion is the process to recover the blood supply after the ischemia. However, in case of interrupted blood supply in brain tissue and after the recovery of blood supply, the function of tissues and organs had not been restored, but aggravated the condition and worsened the function disorder and structural injury [14]. The cerebral blood flow was suddenly increased after the reperfusion and a great number of neutrophils were engorged and attached to the vascular endothelial cells to cause the circulatory disorder of microvessels. The situation of ‘no-reflow’ could reduce the metabolism of brain tissue. With the progression of reperfusion injury, it would cause the cerebral infarction, death of nerve cells and serious damage of neural function [2,15,16]. In this study, the modified NSS of neural function of rats after the cerebral ischemia reperfusion was significantly increased, with the extremely slow recovery of movement, poor balance and reflex and serious situation of drinking and eating. But after the injection of RAC1 activity inhibitor NSC23766, the situation of rats was significantly improved. It indicated that RAC1 might play a certain role in the process of cerebral ischemia reperfusion injury.

In the early stage of cerebral ischemia reperfusion, there might be abundant reactive oxygen to cause the oxidative stress injury, injury of cell function and death of nerve cells [17]. The low molecular weight GTP binding protein RAC1 is the extremely important regulating switch in the whole chain reaction. After being bound with GTP, its activation could activate NADPH to produce the superoxide anions and promote the oxidation process and the generation of oxygen free radicals [18,19]. The previous research reported that the activated RAC1 could promote the generation of reactive oxygen species (ROS). When the activity of RAC1 was inhibited, the generation of ROS would be reduced. The series of reactive oxygen species could regulate the oxidative stress reaction of cells and then the activity of RAC1 could also play the role of regulation in the oxidative stress reaction [20]. In

this study, the expression of RAC1 was significantly increased in rats after cerebral ischemia reperfusion, while RAC1 inhibitor could significantly reduce the expression of RAC1 to relieve the injury because of reperfusion. The study on the oxidative stress indicators also indicated that the application of RAC1 inhibitor could up-regulate the expression of SOD and down-regulate the expression of MDT. As SOD can degrade ROS inside the body to resist the injury by oxidative stress and MDA is the final product of oxidative stress reaction of oxygen free radicals in lipids, it indicates that the inhibition of RAC1 activity can significantly reduce the injury because of oxidative stress reaction.

The impairment of neural function is major measurement criteria of cerebral ischemia reperfusion injury. The survival number of nerve cells and the number of neurons would indicate the degree of impairment of neural function [21]. In this study, Nissl staining was employed to observe the survival of nerve cells after modeling of rats. After the cerebral ischemia reperfusion, the neurons of rats were shrunk and the nerve cells showed the irregular morphology, disorderly arrangement and obvious missing of Nissl bodies. But after the injection of RAC1 inhibitor, the condition of nerve cells was significantly improved, which meant that it could protect the neural function. In conclusion, RAC1 plays a key role in the process of cerebral ischemia reperfusion injury of rats, which can inhibit the activity of RAC1, reduce the level of oxidative stress and relieve the impairment of neural function to protect the neural function.

Conflict of interest statement

We declare that we have no conflict of interest.

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