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Original Article

NSC23766, A Specific Inhibitor of Rac1 GTPase, Attenuated Delayed Neuronal Cell Death and Cognitive Deficit in Rats with Cardiac Arrest

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ABSTRACT

Background: Recent studies have implicated that inhibition of Rac1 GTPase protects the neuronal cells from cerebral ischemic injury, although its effects in a cardiac arrest (CA) global cerebral ischemia model have not been examined. The purpose of the current study was to examine the regulatory and functional role of the Rac1 in reactive oxygen species (ROS) generation and neuronal cell death/cognitive dysfunction following global cerebral ischemia in the rats.

Methods: A rat model of CA was established by the delivery of alternating current between the esophagus and chest wall to induce ventricular fibrillation. Rats were randomly assigned to the following groups: 1) sham group, 2) CA group, 3) CA + NSC23766 group (NSC), and 4) CA + saline group. NSC23766 (50 μg, intracerebroventricular injection) or isotonic saline was administered 15 minutes before CA. Neurological function of rats after reperfusion was scored using the neurological deficit score evaluation method. Survival rates, neuronal counts, Rac1 activation, mitochondrial ROS formation and expression of thioredoxin2 (Trx2) and peroxiredoxin3 (Prx3) in the hippocampal CA1 region were assessed after reperfusion. Morris water maze task was used to quantify spatial learning and memory deficits after reperfusion.

Results: NSC23766, the specific inhibitor of Rac1 GTPase, significantly attenuated neuronal damage and reduced the spatial learning and memory deficits associated with global cerebral ischemia induced by CA ($P<0.05$). Furthermore, NSC23766 exerted its neuroprotective function by decreasing the ROS generation ($P<0.05$). However, there was no significant difference of Trx2 and Prx3 protein expression in hippocampal CA1 region between the CA group and NSC group after 1 day reperfusion ($P<0.05$).

Conclusions: Our findings suggested that NSC23766 not only ameliorated post-ischemic neuronal cell death, but also improved learning and memory deficits in a cerebral ischemia animal model through down-regulating ROS generation.

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In the perioperative period, many accidents can lead to systemic ischemia, such as cardiac arrest (CA), shock and marked hypotension during cardiac surgery, the brain is intrinsically more vulnerable to ischemia than other organs. Furthermore, global ischemia/reperfusion (I/R) damage to the brain, which results in high disability and mortality is associated with oxidative stress (1-3).

During global I/R, oxidative stress is induced by an increase of intracellular concentration of reactive oxygen species (ROS) leading to oxidative damage of DNA, proteins, and other macromolecules, and it ultimately results in cell apoptosis or necrosis (4). Global ischemia following CA can lead to serious cognitive deficits in human and animals. It has been well accepted that the hippocampus which is an important region in learning and memory, particularly, the CA1 region is more vulnerable to oxidative damage than others of the brain (5).

Recently, many studies have demonstrated that a membrane enzyme, NADPH oxidase, plays a critical role in ROS generation, which is triggered by the onset of reperfusion (5, 6). Activation of NADPH oxidase leads to generation of the superoxide ion ($O_2^{\bullet -}$) under pathological conditions, which can quickly transform into highly reactive hydroxide (· OH) and result in a cascade reaction of oxygen radicals (7), contributing to delayed neuronal death in global cerebral I/R injury. NADPH oxidase consists of membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$), cytosolic subunits ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$), and a low-molecular-weight G protein (Rac1 or Rac2) (8). The Rac1 in the guanosine triphosphate (GTP)-bound form (Rac1-GTP) plays a critical role in NADPH oxidase activation after cerebral I/R injury (5). Thus, agents that could suppress Rac1 activation would be expected to protect neuronal cells from oxidative stress-induced damage, and improve cell survival.

NSC23766 has been shown to effectively inhibit Rac1 binding and activation by the Rac-specific guanine nucleotide exchange factors, such as Trio and Tiam1, in a dose-dependent manner (9). Moreover, it has been shown that NSC23766 protects the hippocampus from delayed neuronal cell death following a 4-vessel global cerebral ischemia rat model (5, 10). The present study was designed to address this issue by using a CA global cerebral ischemia rat model and elucidate the underlying signal transduction cascade.

The mitochondrion is another major source of ROS generated during global cerebral I/R. An excessive accumulation of superoxide radials and H_2O_2 serves as a main cause of neuronal damage/death. However, the production of ROS in mitochondria is strictly regulated by mitochondrial antioxidant systems. A vast majority of studies have shown that mitochondrial thioredoxin2 (Trx2)/peroxiredoxin3 (Prx3) redox system which is mitochondria-specific is a major route for removing H_2O_2 in the central nervous system (11, 12). It is also an important factor for cell viability and an essential gene regulating mitochondria-dependent apoptosis.

In this study, we used a transoesophageal electrical stimulation induced CA global cerebral ischemia model to evaluate the effects of intracerebroventricular administration of NSC23766 on neurons and cognitive function of rats. Furthermore, we also investigated the relationship between the neuroprotection of NSC23766 and mitochondrial Trx2/Prx3 redox system.

MATERIALS AND METHODS

Animals and Ethics

Adult male Sprague-Dawley rats (260-300 g), were obtained from the Experimental Animal Centre of Central South University, and used for this study according to the Guide for the Care and Use of Laboratory Animals. The animal experiments were approved by the animal ethics committee of Xiangya Hospital Central South University. Animals were housed in a temperature-controlled (24 ± 1°C) room and maintained under diurnal lighting conditions (12-hour light/dark). They had free access to food and water.

Induction of Global Cerebral Ischemia

The animals were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg body weight), placed in a supine position on a surgical board, and the extremities were immobilized. Tracheal intubation was orally intubated with a 14-gauge catheter under direct vision and fixed at a distance of 6 cm between the distal end of the catheter and the incisor. Electrocar-

diogram (ECG) monitoring was performed using limb leads. A 24 G closed-vein indwelling needle was inserted into right femoral vein to establish transfusion passage. A 22 G closed-vein indwelling needle was inserted into the right femoral artery. The three-way cock was connected to the remaining arterial needle. One end was connected to an injector with heparin, and the other end was connected to the physiological monitoring recorder to monitor arterial blood pressure. An esophageal pacing electrode was implanted in the esophagus of the rat approximately 7.5 cm in depth. We induced ventricular fibrillation (VF) with 6 mA alternating current at a frequency of 50 hertz (Hz), and the wave width is 4 ms. Electrical stimulation was performed for 90 seconds. The electrical stimulation parameters were determined according to our pre-test. CA was identified using the following criteria (13): 1) the systolic arterial pressure after electrical stimulation quickly fell to below 25 mm Hg; 2) pulsations in the arterial pressure waveform disappeared; and 3) the electrocardiographic wave displayed on the cardiac monitor indicated VF, pulseless electrical activity (PEA), or asystole after electrical stimulation stopped. After reaching the criteria for CA, the animals were observed with no intervention for 6 minutes. After 6 minutes of CA, cardiopulmonary resuscitation (CPR) was performed. Rats were resuscitated with epinephrine (20 μg/kg intravenous injection), mechanical ventilation (100% fraction of inspiration O_2 [FiO_2], 60 breathes/minute), and chest compressions (200/minute) performed by the instrument which was made by ourselves. External biphasic defibrillation was performed after 1 minute of CPR if the ECG showed VF. If restoration of spontaneous circulation (ROSC) could not be achieved within 5 minutes, resuscitation efforts were stopped. In successfully resuscitated animals, ventilation rate was adjusted according to the results of blood gas analyses and partial pressure of carbon dioxide (PCO_2) in arterial blood was controlled around 40 mm Hg. The oxygen content decreased gradually to 21-30%, balanced with nitrous oxide (N_2O). They were monitored with the ECG and hemodynamics for 4 hours. During this period, the rats with weak autonomous respirations were mechanically ventilated. Their respiratory condition was evaluated every 15 minutes to determine whether the mechanical ventilation should be continued. Mechanical ventilation was stopped after 4 hours, all tubes were removed and wounds were sutured. Each rat was fed in a separate cage. Body temperature during each experiment was maintained at 37℃ by an infrared heat lamp regulated by feedback from a rectal temperature probe. The indicators of ROSC included recovery of a supraventricular rhythm and a mean arterial pressure $\geqslant 60$ mm Hg that was sustained for >10 minutes.

Experimental Protocols

Rats were randomly assigned to the following groups: 1) sham group, 2) CA group, 3) CA + NSC23766 group (NSC), and 4) CA + saline group (Saline). Arterial and venous catheterization, anesthesia and endotracheal intubation were performed in the sham group. An esophageal electrode was implanted in the sham group with a length of 4 cm from the incisor, and then electrical stimulation using the same parameters was performed for 90 seconds to induce generalized twitching but not CA. In the three CA groups, VF was induced for 6 minutes and then standard CPR was performed. Animals in the NSC group were given NSC23766 (Tocris Bioscience, Ellisville, MO, USA), which was dissolved in 10 μl saline and administered bilaterally into the lateral cerebral ventricles of rats 15 minutes before ischemia for a total dose of 50 μg. The dose of Rac1 GTPase inhibitor was determined on the basis of previous studies (5, 10). Rats in Saline group received bilateral saline injections. The survival duration after resuscitation was observed until 9 days. At 24, 72 hours and 9 days after ROSC, the neurological function of rats was scored using the neurological deficit score (NDS) evaluation method (14). Rats were tested for coordination, motor and sensory function (Table 1) (14). NDS was determined by an examiner blinded to the groups. The scores of NDS range from 0 (brain dead) to 80 (normal). Rats with the greatest neurological deficits died or were sacrificed between 6 hours and 9 days after resuscitation.

Nissl Staining

For hippocampal neuronal survival analysis, the

Table 1. Neurodeficit Scoring for Rats (Normal=80; Brain Dead=0).	
General behavioral deficit	Total score: 19
Consciousness	Normal 10/stuporous 5/comatose or unresponsive 0
Arousal	Eyes open spontaneously 3/eyes open to pain 1/no eye opening 0
Respiration	Normal 6/abnormal (hypo or hyperventilation) 3/absent 0
Brain-stem function	Total score: 21
Olfaction: response to smell of food	Present 3/absent 0
Vision: head movement to light	Present 3/absent 0
Pupillary reflex: pupillary light reflex	Present 3/absent 0
Corneal reflex	Present 3/absent 0
Startle reflex	Present 3/absent 0
Whisker stimulation	Present 3/absent 0
Swallowing: swallowing liquids or solids	Present 3/absent 0
Motor assessment	Total score: 6
Strength	Normal 3/stiff or weak 1/no movement or paralyzed 0 (Left and right side tested and scored separately)
Sensory assessment	Total score: 6
Pain	Brisk withdrawal with pain 3/weak or abnormal response (extension or flexion posture) 1/no withdrawal 0 (Left and right side tested and scored separately)
Motor behavior	Total score: 6
Gait coordination	Normal 3/abnormal 1/absent 0
Balance on beam	Normal 3/abnormal 1/absent 0
Behavior	Total score: 12
Righting reflex	Normal 3/abnormal 1/absent 0
Negative geotaxis	Normal 3/abnormal 1/absent 0
Visual placing	Normal 3/abnormal 1/absent 0
Turning alley	Normal 3/abnormal 1/absent 0
Seizures (convulsive or non-convulsive)	Total score: 10 No seizure 10/focal seizure 5/general seizure 0

Balance beam testing was normal if the rat can cross a 2 cm wide by 1 m long beam suspended 0.5 m above the floor. Abnormal was scored if the rat attempted and did not continue or stayed momentarily and fell. Absent was scored when the rat fell off immediately upon placement on the beam. Other behavior reflex subscores evaluated the following: 1) righting reflex: animal placed on its back was able to correct to upright position; 2) turning alley: the animal was made to walk and turn back at the end of a 15 cm × 0.5 m alley; 3) visual placing: the animal was lifted and was able to visually orient itself to objects and depth; 4) negative geotaxis: animal placed on its back on a plane angled at 45° corrected itself and moved up the incline.

rats that survived for 9 days and non- arrested controls were deeply anesthetized with chloral hydrate and transcardially perfused with 200 ml of normal saline, followed by 300 ml of 4% paraformaldehyde in 0.1 mmol/L phosphate buffered saline at 4° C (pH 7.4). Brains were quickly removed and fixed in 4% paraformaldehyde at 4°C for an additional 24 hours. After fixation, the brains were embedded in paraffin. Serial 5 μm coronal sections were made at the level of anterior hippocampus and nissl staining. The entire length of the hippocampal pyramidal cell layer in the hippocampus was viewed under high power light microscopy (400 ×). Neurons with rounded cell bodies and clearly visible nucleoli were counted. The number of neuronal surviving was evaluated in CA1 region of pyramidal cells in hippocampus.

Isolation of Mitochondria

After ROSC of 6 hours, rats were deeply anesthetized with chloral hydrate and decapitated. The brains were quickly removed, and the hippocampal CA1 region was dissected on ice. The cytosolic and mitochondrial fractions of the harvested brain tissues were prepared as previously described (15). Each brain tissue sample was homogenized in ice- cold lysis buffer (200 mM

mannitol, 80 mM N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid [HEPES]-potassium hydroxide [KOH] [pH 7.4], and the protease inhibitor cocktail) by 35 strokes of gentle pounding in a glass tissue grinder. Homogenates were centrifuged at 750×g for 10 minutes at 4℃. After removing a portion of the supernatant, the remaining supernatant was centrifuged at 8,000×g for 20 minutes at 4℃. The mitochondrial fraction was obtained after washing the resultant pellets three times and resuspending the pellet in lysis buffer. The cytosolic fraction was obtained by centrifuging the supernatants of the 8,000×g centrifugation step at 100,000×g for 1 hour at 4℃.

Rac1 Activation Assay

Assays of Rac1 activation were performed using p21-activated kinase (PAK1)-p21-binding domain (PBD) color agarose beads according to the manufacturer's protocol (Rac1 Activation Assay Kit, Cell Biolabs). Briefly, 400 μg samples were mixed with 40 μl of PAK1-PBD agarose beads and incubated for 1 hour at 4℃. The reaction was terminated by addition of $MgCl_2$. The agarose beads were collected by spinning at 14,000×g for 30 seconds at 4℃ and the supernatants removed. Wash the bead 3 times with Assay Buffer and boil it in sample buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane, and detected by immunoblotting using an anti-Rac1-specific antibody (1:1000, Cell Biolabs).

Measurement of ROS

ROS formation was detected with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent probe, according to the instruction of ROS assay kit (Beyotime Institute of Biotechnology, China) with slightly improvement. In brief, 10 μl of mitochondria (5-10 μg) was suspended in 170 μl HEPES buffer (120 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 5.0 mM $NaHCO_3$, 6.0 mM glucose, 1.0 mM $CaCl_2$, 10 mM HEPES, pH 7.4) in 96-well plates; 20 μl of 100 μM DCFH-DA was added to each well, for a final volume of 200 μl. The fluorescence was then measured at 488 nm excitation and 525 nm emissions by a fluorescent plate reader (Genios, TECAN). Increased fluorescence intensity was considered to be indicative of an increase in intracellular ROS.

Western Blotting Analysis

In order to examine the protein levels of antioxidants in the ischemic CA1 region, the animals that survived for 1 days and non-arrested controls were used for western blot analysis, according to previous study. Equal amounts of protein were loaded onto a suitable polyacrylamide gel. After electrophoresis, separated proteins were transferred to nitrocellulose transfer membranes (PVDF; Millipore, Bedford, MA, USA) and blocked in 5% non-fat dry milk prepared in 1X tris buffered saline with tween 20 (TBST), followed by incubation with Trx2 (diluted 1:200, Santa Cruz, CA, USA), Prx3 (diluted 1:3000, Abcam, Cambridge, MA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1: 1,000, Cell Signaling Technology, Beverly, MA, USA) overnight at 4℃. Subsequently, the membranes were washed with 1X TBST and the membranes incubated with appropriate secondary antibodies for 2 hours at room temperature. The blots were developed using an enhanced chemiluminescence (ECL) plus detection system (Beyotime Institute of Biotechnology, China) and relative band density measured using the FluorChem FC2 System (NatureGene Corp., Medford, NJ, USA).

Morris Water Maze

To evaluate spatial learning and memory, rats were placed in a circular pool (1.6 m in diameter and 60 cm high). A platform (12 cm) submerged 2.0 cm beneath the surface of water (25 ± 1℃). An automatic tracking system was used to record escape latency and the swimming path. The water maze, which was conceptually divided into four quadrants, was located in a quiet room that was decorated with visual cues. Rats were placed into the maze at different starting positions and received training of searching an unmarked platform composing of 4 trials per day with maximum trial duration of 120 seconds and 30 seconds on platform at the end of trials. In the probe trial performed 24 hours after the last training session, the platform was re-

Table 2. Survival Rates (9 Days) after CA and Global Cerebral Ischemia.

Experimental group	Number of deaths					Survival rate
	<1 day	1-2 days	2-3 days	3-4 days	4-9 days	9 days/total
Sham	0	0	0	0	0	100% (0/18)
CA	9	7	3	0	0	47.2% (17/36)
NSC	6	4	2	0	0	66.7% (24/36)*
Saline	9	6	4	1	0	44.4% (16/36)

*Indicates significantly different from the CA group (Wilcoxon [Gehan] survival analysis, P<0.05).

moved and the memory of platform position was evaluated by putting a rat to opposite quadrant to platform quadrant. The percent of time spent in the target quadrant, which previously contained the platform, and the number of times the rat crossed over the exact location of the former platform, were recorded for each rat. After testing, the rats were sacrificed for Nissl staining.

Statistical Analysis

Data were expressed as means ± standard deviation (SD). Normal distribution for the variable was tested by Kolmogorov-Smirnov test and all variables were normally distributed. Group differences in escape latency in the Morris water maze task were analyzed using a two-way analysis of variance (ANOVA) with repeated measures. The two factors of this analysis were group and training day. Other data were analyzed using a one-way ANOVA with the Student-Newman- Keuls or Dunnett's test (SPSS16.0, Inc., Cary, NC, USA). $P<0.05$ was considered statistically significant.

RESULTS

Overall Survival Rates after CA and Global Cerebral Ischemia

As seen in table 2, 9-day overall survival rates were determined in resuscitated rats of all the groups. At the end of 9 days, the rats treated with NSC23766 had a significantly better (66.7%, 24/36, $P<0.05$, Wilcoxon [Gehan] survival analysis) survival rate compared to the CA rats (47% , 17/36) and Saline- treated group (44%, 16/36). Of those that did not survive for 9 days, most died during the first 3 days after resuscitation, suffering mostly from respiratory failure or cardiovascular collapse.

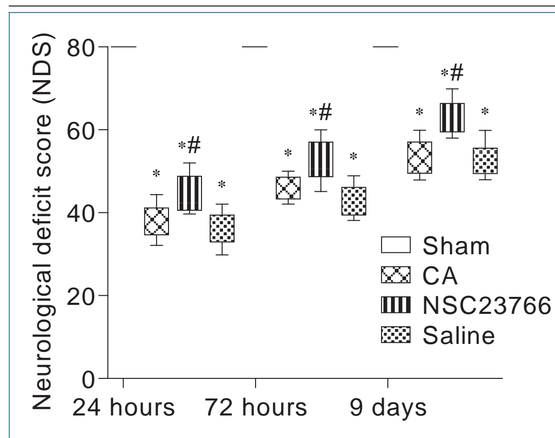

Figure 1. NDS was Observed at 24, 72 Hours and 9 Days after ROSC.
*P<0.05 vs. sham group; #P<0.05 vs. CA and Saline groups.

Effects of NSC23766 on Neurological Deficits

The NDS were evaluated after 24, 72 hours and 9 days of resuscitation (Figure 1). Sham-operated rats did not have any deficits. However, animals in CA and Saline groups developed severe neurological deficits after 24, 72 hours and 9 days of resuscitation, respectively. In contrast, the score improved in NSC23766-treated group after 24, 72 hours and 9 days of resuscitation, compared with CA and Saline group.

NSC23766 Attenuated Hippocampal Neurons from I/R Injury

The hippocampal neuronal cell counts were determined at 9 days of recovery from 6 minutes CA in all groups. On Nissl- stained sections, which was measured in 9 days after resuscitation, many atrophic neurons with shrunken cytoplasm and damaged nuclei were observed in the rats of CA and Saline groups, while there is no apparent morphological changes in corresponding position of sham group as control. Hence, NSC23766 significantly prevented the neuron

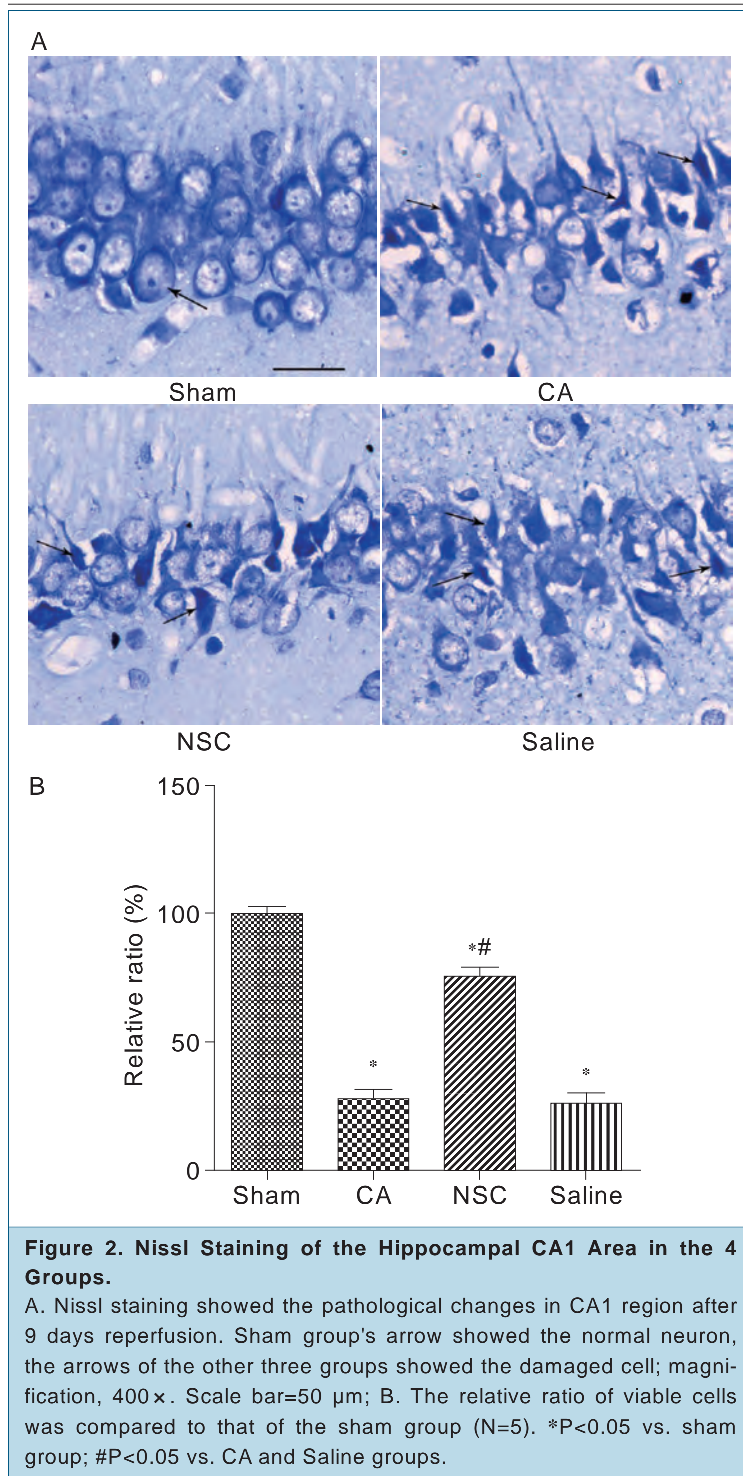

Figure 2. Nissl Staining of the Hippocampal CA1 Area in the 4 Groups.
A. Nissl staining showed the pathological changes in CA1 region after 9 days reperfusion. Sham group's arrow showed the normal neuron, the arrows of the other three groups showed the damaged cell; magnification, 400×. Scale bar=50 μm; B. The relative ratio of viable cells was compared to that of the sham group (N=5). *P<0.05 vs. sham group; #P<0.05 vs. CA and Saline groups.

loss compared with that in CA and Saline groups (Figure 2, $P<0.05$).

NSC23766 Decreased the Activities of Rac1 after Reperfusion

As shown in figure 3, western blot analysis revealed that total Rac1 protein levels in hippocampus did not change significantly at any groups examined at 6 hours after reperfusion. The time point was chosen according to our pre-experiment, which observed that the activation of Rac1 GTPase reached the peak levels at 6 hours after global cerebral I/R. In contrast, activated Rac1 (Rac1-GTP) showed a marked elevation in CA and Saline groups, as compared to sham controls. Administration of NSC23766 significantly reduced activated Rac1.

Mitochondrial ROS Formation

Rac1 GTPase activation has been suggested to be critical for NADPH oxidase activation and $O_2^{\bullet-}$ production in the brain. To explore this possibility, we examined the ROS production in the rat hippocampal CA1 region following global cerebral I/R at 6 hours. We found that administration of NSC23766 leaded to a reduction in ROS production. As shown in figure 4, compared with sham group, mitochondrial ROS accumulation in CA and Saline groups were significantly increased. However, ROS production levels were significantly decreased in the hippocampal mitochondria of the NSC23766 treatment group, when compared to those of the vehicle group.

Morris Water Maze

To investigate whether administration of NSC23766 reduced neuronal damage and also improved behavioral deficits, we tested the spatial learning and memory of rats in the Morris water maze task. There were significant differences in mean escape latency between CA and NSC groups in 4 training days. Compared with rats from the sham group, the three CA groups took longer to find the hidden platform on all training days. Concerning spatial learning ability, no significant difference between the CA and Saline groups was detected at any stage of the training. In the probe trial, the NSC group made much more platform crossings than either CA or Saline group did. Differences between the CA and Saline groups were not significant. Finally, the CA and Saline groups spent less time in the target quadrant as compared with either the sham or NSC group. A significant difference was observed between the CA and NSC groups (Figure 5).

Changes in Antioxidants Levels

In the present study, the levels of Trx2 and Prx3 proteins were examined after 1 day reperfusion to compare their changes in the CA1 region of 4 groups. The levels of the antioxidants in the sham group were lower than those in the three CA groups. However, there were no significant differences between CA group and NSC group (Figure 6).

DISCUSSION

In this study, NSC23766, the specific inhibitor of Rac1 GTPase, significantly attenuated neuronal damage and reduced the spatial learning and memory deficits associated with global cerebral ischemia induced by CA. Finally, the rats treated with NSC23766 had a better survival rate.

As we all known, mortality and morbidity from global cerebral ischemia which are primarily due to I/R injury are a significant public health problem. CA is one of the most prevalent reasons of global cerebral ischemia in clinical practice, mainly caused by malignant arrhythmia (16). Compared with other methods, in experimental animals, the arrhythmia is well simulated by transoesophageal electrical stimulation of heart (17, 18). Moreover, this model is easier to perform, less harmful and more reproducible than others of CA.

Free radicals play a vital role in global cerebral I/R injury. Previous studies have showed that NADPH oxidase is a major complex that produces oxygen-derived free radicals during cerebral I/R periods (6, 19, 20). Rac1 is a key subunit of NADPH oxidase and is required for NADPH oxidase assembly and activation (21). Thus, Rac1 usually exerts its function through NADPH oxidase and ROS production in neurons. Growing evidences have indicated that ROS from NADPH oxidase contributes to neuronal cell damage in response to global cerebral I/R. In this regard, we used the specific inhibitor of Rac1 GTPase, NSC23766. NSC23766 was shown to effectively inhibit Rac1 binding and activation by the Rac-specific guanine nucleotide exchange factors (GEF) such as Trio and Tiam1 in a dose-dependent manner (9). Furthermore, previous studies have shown that NSC23766 protects the hippocampus from delayed neuronal cell death following a 4-vessel global cerebral ischemia rat model (5, 10). However, the present study described and investigated the neuroprotective role of NSC23766 after global cerebral ischemia using a CA model. Our results demonstrated that NSC23766 significantly attenuated the activation of Rac1 of the hippocampus after reperfusion, compared to CA and Saline groups.

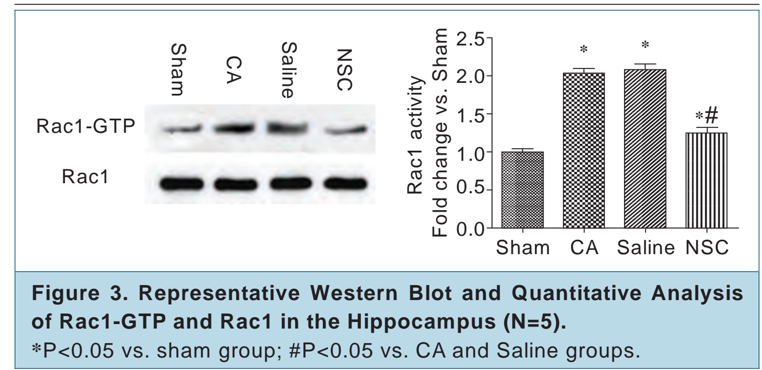

Figure 3. Representative Western Blot and Quantitative Analysis of Rac1-GTP and Rac1 in the Hippocampus (N=5).
*P<0.05 vs. sham group; #P<0.05 vs. CA and Saline groups.

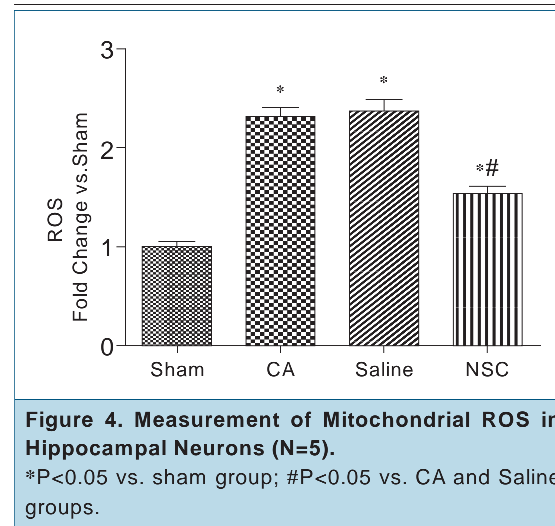

Figure 4. Measurement of Mitochondrial ROS in Hippocampal Neurons (N=5).
*P<0.05 vs. sham group; #P<0.05 vs. CA and Saline groups.

The brain is intrinsically vulnerable to global ischemia, especially in the CA1 region of the hippocampus, which is closely related to cognitive dysfunction after I/R injury (22, 23). The current study found that administration of NSC23766 prior to ischemia effectively reduced neuronal damages in CA1 region and improved spatial learning and memory induced by global cerebral I/R. Finally, the rats treated with NSC23766 had a better survival rate. Nissl staining revealed various degrees of neuronal degeneration and necrosis in rats after 6 minutes of global ischemia, and the degree of

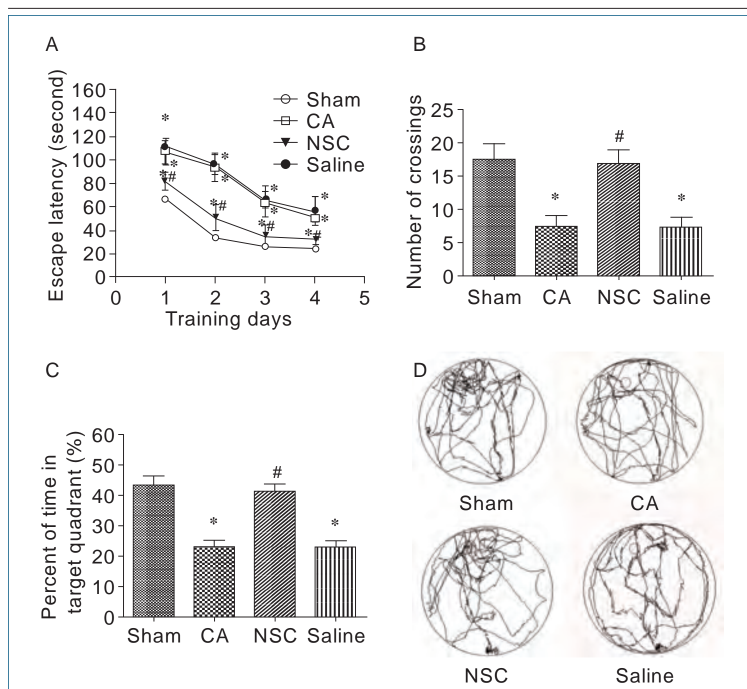

Figure 5. The Morris Water Maze was Performed to Test the Spatial Learning and Memory in the Sham, CA, NSC, Saline Groups from the 5 to 9 Days after Reperfusion.
A. Escape latency to find the hidden platform during four consecutive days of training (N=5); B. The number of times the rats crossed over the former platform location during the probe trial (N=5); C. The percent of time spent in the target quadrant during the probe trial (N=5); D. Representative swimming paths during the probe trial for each group (N=5). *P<0.05 vs. sham group; #P<0.05 vs. CA and Saline groups.

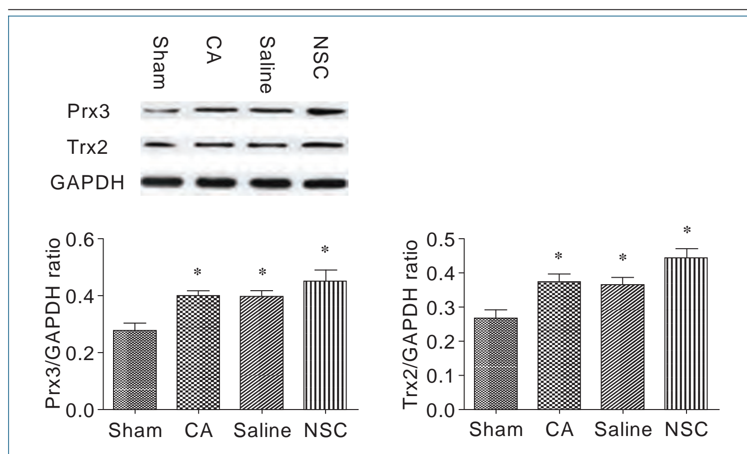

Figure 6. Representative Western Blot (Upper) and Quantitative Analysis of Prx3 (Under, Left) and Trx2 (Under, Right) in the Hippocampus of Rats after Global Ischemia/Reperfusion (N=5).
*P<0.05 vs. sham group.

pathological changes was consistent with the NDS and survival rate. We found lots of degenerative and necrotic neuronal cells in CA and Saline groups, and few were detected in NSC group. This showed that NSC23766 effectively protected the neuronal cells from global cerebral I/R injury of a CA model. In addition, the results of Morris water maze task demonstrated that 6 minutes of global ischemia significantly impaired spatial learning and memory. NSC23766 decreased the escape latency, increased the number of platform site crossings, and increased the time spent in the target quadrant. It seemed, therefore, that neurons rescued by the NSC23766 pretreatment maintained at least some of their functional parameters. The performance of the Rac1 GTPase inhibitor treated animals on the Morris water maze was also reflective of the attenuation of oxidative stress and enhanced neuronal survival in the hippocampal CA1 region of NSC23766 treated rats as compared to saline treated (ischemia) rats.

It is likely that Rac1 GTPase activation contributes to multiple pathological events, which collectively facilitate neuronal damage and cognitive dysfunction following global cerebral ischemia. The present study showed that inhibition of Rac1 GTPase activation markedly decreased neuronal damage to hippocampal CA1 region following global I/R injury, which was likely due to a correlated significant attenuation of $O_2^{\bullet -}$ generation. Superoxide is the direct product of NADPH oxidase activation and is the first ROS type in the oxygen free radical chain (24). Various cascade reactions then convert $O_2^{\bullet -}$ into H_2O_2, $OH^{\bullet -}$, or peroxynitrite ($OONO^-$) (25), which cause cellular injury by protein oxidation, lipid peroxidation, and DNA breakage. Mitochondrion is another important source of ROS production contributing to I/R injury. Recent studies have shown that mitochondrial Trx2 and Prx3, which are major compartments in the mitochondrial Trx/Prx redox system, detoxify H_2O_2 in the central nervous system (11, 12, 26). They exert important roles in antioxidant regeneration and regulation of intracellular ROS level by converting H_2O_2 to water and oxygen (11, 27, 28). Thus, Prx3/Trx2 provided a substantial neuroprotective effect against ischemic damage by reducing the oxidative stress (29). Previous studies also reported that mitochondrial ROS ac-

tivates phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC). Activation of PI3K in turn promotes Rac1 and subsequent NADPH oxidase activation (30, 31). So, there is a mutual functional relationship between NADPH oxidase and mitochondria in a feed-forward fashion, which promotes neuronal cells damage after cerebral I/R. Thus, we examined whether there are some connections between the neuroprotection of NSC23766 and mitochondrial Trx2/Prx3 redox system. In our experiment, Trx2 and Prx3 protein levels were significantly increased in the three CA groups after 1 day reperfusion. The increase of Trx2 and Prx3 in hippocampal CA1 region at 1 day post-ischemia may be associated with compensatory mechanisms against ischemic damage. However, inhibiting the activation of NADPH oxidase did not lead to significant differences in the expression of Trx2 and Prx3 between NSC and CA groups after 1 day of reperfusion. It is possible that Rac1 exerts its function only trough NADPH oxidase/ROS signaling in the CA global cerebral ischemia model rather than the mitochondrial Trx/Prx redox system, or we need to examine more time points after reperfusion.

Nevertheless, our study had several limitations. Firstly, in the present study, the long-term survival rate was not observed. Secondly, it was unclear whether NSC23766 administered immediate or after the CA was similarly protective. Since it is significant in clinical. Therefore, further studies are required to address these important issues.

In conclusion, this experiment has shown that inhibition of Rac1 GTPase attenuated neuronal cell damage and cognitive dysfunction in a CA global cerebral ischemia model and the rats treated with NSC23766 had a better survival rate. Thus, decreasing Rac1 activation may be a useful therapy for global cerebral I/R injury.

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