Protective effects of notoginsenoside R1 on cerebral ischemia-reperfusion injury in rats

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Abstract. The objective of this study was to investigate the protective effect of notoginsenoside R1 (NGR1) on cerebral ischemia-reperfusion injury (CIRI) in rats, and its molecular mechanism, to provide new insights into the diagnosis and treatment of CIRI. Sixty Sprague-Dawley rats were randomly divided into four groups including the sham-operation group (Sham), cerebral ischemia-reperfusion model group (CIR), NGR1 treatment group (NGR1), and nimodipine positive control group (NDC) with 15 rats each. Bilateral common carotid arteries occlusion was used to establish the rat CIRI model. The area of cerebral infarction at the end of reperfusion was calculated by triphenyl tetrazolium chloride staining. Apoptosis of hippocampal neurons in each group was detected by Annexin V/propidium iodide double staining. Hippocampal expression of brain-derived neurotrophic factor (BDNF) mRNA, and Bcl-2 and Bax protein at the end of reperfusion were measured by RT-qPCR and western blot analysis, respectively. Data were analyzed by SPSS software analysis to ensure statistical significance. At the end of reperfusion, the area of cerebral infarction in the NGR1 and NDC groups was significantly smaller than that of the CIR group. Apoptosis analysis showed that compared with the CIR group, the apoptosis rate of hippocampal neurons was significantly decreased in the NGR1 and NDC groups. RT-qPCR and western blot analysis showed that at the end of reperfusion, higher levels of BDNF mRNA and the anti-apoptotic factor, Bcl-2, and lower levels of the pro-apoptotic factor, Bax, in the hippocampus were found in the NGR1 and NDC groups compared with the CIR group. The protective effect of NGR1 on CIRI was significantly stronger than that of nimodipine. In conclusion, NGR1 can reduce the area of cerebral infarction, reduce apoptosis of hippocampal neurons, and protect rats from CIRI. Those effects were achieved by activating the expression of BDNF and Bcl-2, and by inhibiting the expression of Bax.

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Introduction

Cerebral ischemia, also known as cerebral apoplexy, is a cerebrovascular disease characterized by reduction in cerebral blood flow. Cerebral ischemia accounts for 85% of all cases of stroke, and is the second major cause of disability and death (1). At present, the most effective way to treat cerebral ischemia is intravenous injection of plasminogen activator for thrombolysis. However, this treatment strategy is often accompanied by cerebral ischemia-reperfusion injury (CIRI). CIRI refers to aggravated nerve injury and dysfunction after reperfusion in patients with cerebral ischemia. The mechanism of CIRI is related to oxidative stress, inflammation, calcium overload, and other factors (2,3), among which oxidative stress is caused by excessive production of reactive oxygen species (ROS), which plays an important role in the pathogenesis of ischemic stroke by exacerbating brain damage (4). Antioxidative therapy against ROS, which can prevent neuronal oxidative damage, may represent a promising method in the radical treatment of ischemic stroke. Clinical trials showed that application of antioxidants that can clear ROS failed to achieve satisfactory results (5). Therefore, novel drugs that can effectively treat CIRI are needed.

Panax notoginoside (PNS), a valuable traditional Chinese herbal medicine, is a phytoestrogen isolated from the dried roots of the perennial herb, Panax notoginseng. The main active ingredients of PNS include ginsenoside Rb1, Rg1, and notoginsenoside R1 (NGR1) (6). Numerous studies have shown that PNS has therapeutic effects on various neurodegenerative disorders, and the use of PNS during global cerebral ischemia-reperfusion and focal ischemia-reperfusion can significantly reduce cerebral edema. PNS also has a satisfactory effect on acute cerebral infarction (6). Neuronal apoptosis plays an important role in the pathogenesis of CIRI. Studies have shown that PNS can inhibit the expression and activation of caspase-3 by regulating the expression of Bcl-2 family genes to reduce neuronal apoptosis caused by CIRI. This results in increased survival of neurons after cerebral ischemia (7). In addition, ginsenoside Rb1 and Rg1 have neuroprotective effects on mice with CIRI (8). In recent years, studies have reported that NGR1 can regulate various biological processes,

such as antioxidation, anti-inflammation, and anti-apoptosis, and has neuroprotective activities (9).

Brain-derived neurotrophic factor (BDNF) is a nerve growth factor that is widely distributed in brain tissue. BDNF can affect neurons by significantly increasing the expression of its receptor, TrkB, which is expressed on the surface of neurons. BDNF can also inactivate injury factors in cells by activating TrkB (10). Studies have shown that cerebral ischemia and CIRI can increase the expression of BDNF, thereby enhancing the ability of local neurons to resist injury, to protect the patients from CIRI (11). The aim of this study was to investigate the protective effect of NGR1 on rats with CIRI, and its molecular mechanism, to provide a theoretical basis and potential molecular target for the treatment of CIRI.

Materials and methods

Experimental animals. Sixty specific pathogen-free grade adult male Sprague-Dawley rats (250-300 g) were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Animals were kept at 23-25˚C with relative humidity of 45-55% and light cycle of 12 h. Rats were allowed free access to food and water. They were randomly divided into four groups including the sham-operation group (Sham), cerebral ischemia-reperfusion model group (CIR), NGR1 treatment group (NGR1), and nimodipine positive control group (NDC), with 15 rats in each group. All animal experiments were carried out strictly in accordance with the Guidelines of Proper Care and Use of Laboratory Animals in Research established by the National Institute. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Kunming Medical University (Yunnan, China).

Reagents. NGR1 (purity >98%; Shanghai Ronghe Pharmaceutical Technology Development Co., Ltd., Shanghai, China); Nimodipine (Shandong Xinhua Pharmaceutical Co., Ltd., Shandong, China); chloral hydrate and triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA); Annexin V/propidium iodide (PI) cell apoptosis detection kit (Invitrogen, Carslbad, CA, USA); rabbit anti-rat Bcl-2, Bax, and β-actin primary monoclonal antibodies (cat. nos. 3498, 2772 and 8457), and HRS goat anti-rabbit secondary polyclonal antibody (cat. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA); Modified BCA kit (Sangon, Shanghai, China); TRIzol reagent, Prime Script® RT reagent kit with gDNA Eraser and SYBR® Premix Ex Taq™ II (Takara, Liaoning, China). All primers were synthesized by Sangon.

Establishment of the rat model of CIRI. The rat model of CIRI was established using the bilateral common carotid artery occlusion (BCCAO) method described by Schmidt-Kastner *et al* (12). All surgical tools were autoclaved in advance, and the entire procedure was carried out in a sterile environment. Rats were fasted at 6 h before surgery. After anesthesia by intraperitoneal injection of chloral hydrate at a dose of 350 mg/kg, a surgical blade was used to make an incision in the middle of the neck. At the bilateral common carotid artery, the surrounding tissue and vagus nerves were quickly and carefully separated and exposed. Next, the bilateral common carotid artery was ligated with two 5-0 threads, and the threads were removed 20 min later to restore cerebral blood perfusion. Surgical incisions were sutured, and rats were placed in a 37˚C incubator to allow their body temperature to return to normal. Rats were transferred to cages when they were awakened and righting reflex was restored.

Animal grouping and drug treatment. Sham: The bilateral common carotid artery was exposed but not ligated, and the incision was sutured using a conventional method; CIR: Rats were treated with BCCAO surgery, the bilateral common carotid artery was ligated and reperfusion was performed for 3 h, 20 min later, followed by intragastric administration of 0.5 ml saline; NGR1: Rats were treated with BCCAO surgery, the bilateral common carotid artery was ligated, and reperfusion was performed for 3 h, 20 min later, followed by intragastric administration of NGR1 at a dose of 100 mg/kg; NDC: Rats were treated with BCCAO surgery, the bilateral common carotid artery was ligated, and reperfusion was performed for 3 h, 20 min later, followed by intragastric administration of nimodipine at a dose of 1 mg/kg.

Determination of cerebral infarction area. Three rats from each group that recovered (24 h after surgery) were treated with excessive urethane for euthanasia. The neck of rats was cut, and the brain was harvested after craniotomy, and stored at -80˚C for 15 min. After, brain tissue was sectioned with a thickness of roughly 2 mm followed by staining in 2% TTC solution at 37˚C for 30 min, and soaking in 10% paraformaldehyde solution overnight. Living brain tissue was stained brick red in color, while the areas of infarction were stained gray. The percentage of cerebral infarction area to total brain tissue area was calculated using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Analysis of hippocampal neuron apoptosis. Analysis of hippocampal neuron apoptosis was performed according to the instructions of the Annexin V/PI cell apoptosis assay kit. Three rats returned to normal state in each group (24 after surgery) were used to isolate hippocampal neurons. After washing twice with precooled PBS, hippocampal neurons were incubated with 100 μl 1X binding buffer containing 5 μl Annexin V and 1 μl PI for 15 min. After, 400 μl 1X binding buffer was added and apoptosis was analyzed immediately using FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Real-Time qPCR analysis. Three rats returned to normal state in each group (24 after surgery) were used to isolate total RNA according to the instructions of the kit. After cDNA synthesis by reverse transcription, the reaction system for real-time PCR was prepared, followed by PCR on a CFX-96 Real-Time PCR Detection system (Bio-Rad Laboratories, New York, NY, USA) to measure the expression of BDNF mRNA. All data were processed using the $2^{-\Delta\Delta Cq}$ method with the sham group as the control group, and β-actin as the endogenous control. The primer sequences are shown in Table I.

Western blot analysis. Three rats returned to normal state of each group (24 after surgery) were used to isolate hippocampal tissue. The tissue was stored in liquid nitrogen before

Table I. Sequences of primers used in real-time qPCR.

Gene	Primer sequences (5'-3')
BDNF-F	CTGGAGAAAGTCCCGGTAT
BDNF-R	GGTAGTCGGCATFGCGAGT
ACTB-F	CAGGGCGTGATGGTGGGCA
ACTB-R	CAAACATCATCTGGGTCATCTTCTC

F, forward; R, reverse.

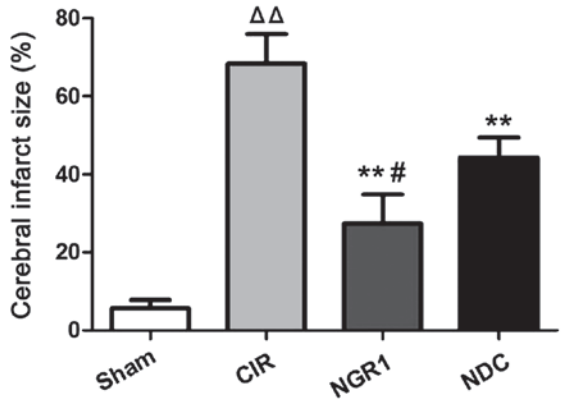

Figure 1. Area of cerebral infarction in each group at 24 h after surgery. TTC staining showed that compared with the Sham group, the area of cerebral infarction in the CIR group was significantly enlarged, while the cerebral infarction area was significantly smaller in the NGR1 and NDC groups compared with the CIR group. Compared with the NDC group, the cerebral infarction area was significantly smaller in the NGR1 group. $^{\Delta\Delta}$Compared with the Sham group, p<0.01; **compared with the CIR group, p<0.01; $^{\#}$compared with the NDC group, p<0.05. TTC, triphenyl tetrazolium chloride; CIR, cerebral ischemia-reperfusion model group; NGR1, notoginsenoside R1 treatment group; NDC, nimodipine positive control group.

use. Hippocampal tissue was homogenized in cell lysis buffer, followed by centrifugation at 4˚C (2,500 x g for 10 min) to collect the supernatant. Protein concentration was determined by a modified BCA kit. A total of 50 μg of protein from each sample was subjected to 10% SDS-PAGE, followed by electrotransfer to PVDF membranes. After blocking with TBST buffer containing 5% skim milk at room temperature for 2 h, membranes were incubated with rabbit anti-rat Bcl-2, Bax, or β-actin primary monocolonal antibodies (1:1,000) overnight at 4˚C. After washing three times with 0.05% TBST, the membranes were incubated with HRL goat anti-rabbit secondary polyclonal antibody (1:2,000) at room temperature for 1 h under vibration. An ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the signals, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the gray value of each band. Relative protein levels are presented as the percentage of the value of the target protein to that of β-actin.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). All experiments were repeated three times, and data are presented as mean ± standard deviation. Single factor analysis of variance and two-tailed t-test were performed for comparisons between groups. p<0.05 was considered to indicate a statistically significant difference.

Results

Effects of NGR1 treatment on cerebral infarction area. The area of cerebral infarction at 24 h after surgery was measured by TTC staining. As shown in Fig. 1, compared with the Sham group, the area of cerebral infarction in the CIR group was significantly enlarged, while the cerebral infarction area was significantly smaller in the NGR1 and NDC groups compared with the CIR group. Compared with the NDC group, the cerebral infarction area was significantly smaller in the NGR1 group. These data suggest that NGR1 can significantly reduce the area of cerebral infarction after CIRI in rats, and this effect is stronger than that of the positive control.

Effects of NGR1 treatment on apoptosis of hippocampal neurons. Apoptosis of hippocampal neurons was detected by the Annexin V/PI double staining method. As shown in Fig. 2, the upper right quadrant scatterplot represents apoptotic cells. Compared with the Sham group, the ratio of apoptotic hippocampal neurons was significantly increased, while the ratio of apoptotic hippocampal neurons was significantly lower in

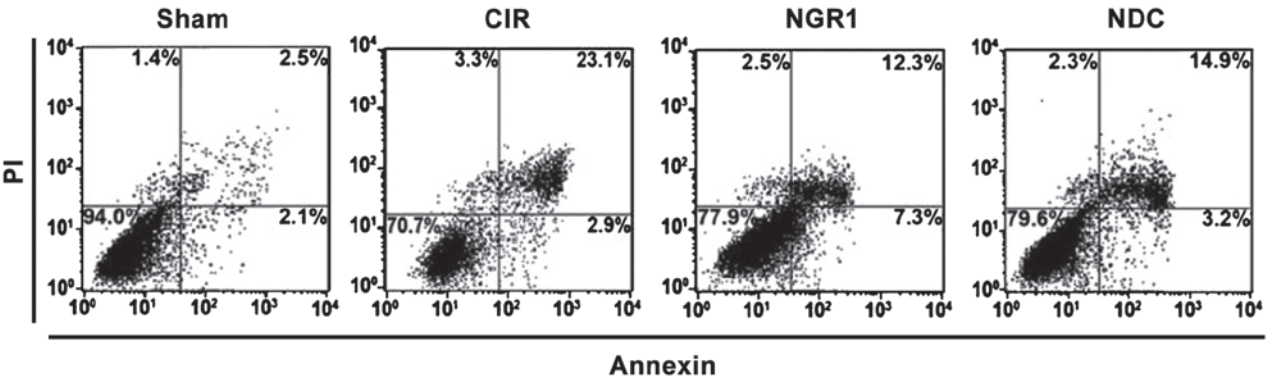

Figure 2. Apoptosis of hippocampal neurons in each group at 24 h after surgery. Apoptosis analysis showed that compared with the Sham group, the ratio of apoptotic hippocampal neurons was significantly increased, while the ratio of apoptotic hippocampal neurons was significantly decreased in the NGR1 and NDC groups compared with the CIR group. In addition, the ratio of apoptotic hippocampal neurons was significantly lower in the NGR1 group compared with the NDC group. NGR1, notoginsenoside R1 treatment group; NDC, nimodipine positive control group; CIR, cerebral ischemia-reperfusion model group.

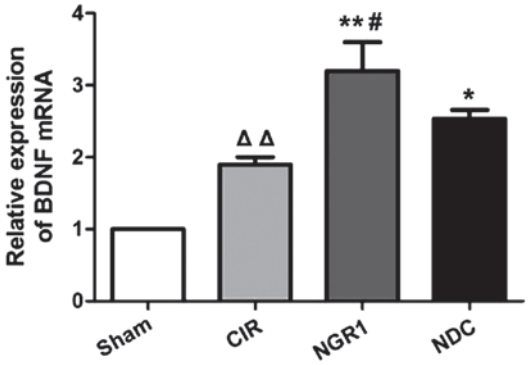

Figure 3. Relative hippocampal expression of BDNF mRNA in each group at 2 h after surgery. qRT-PCR showed that compared with the Sham group, the expression of BDNF mRNA was significantly increased in the CIR group, while the expression of BDNF mRNA was significantly higher in the NGR1 and NDC groups compared with the CIR group. In addition, the expression of BDNF mRNA was significantly higher in the NGR1 group compared with the NDC group. $^{\Delta\Delta}$Compared with the Sham group, $p<0.01$; **compared with the CIR group, $p<0.01$; $^{\#}$compared with the NDC group, $p<0.05$. BDNF, brain-derived neurotrophic factor; CIR, cerebral ischemia-reperfusion model group; NGR1, notoginsenoside R1 treatment group; NDC, nimodipine positive control group.

the NGR1 and NDC groups compared with the CIR group. Compared with the NDC group, the ratio of apoptotic hippocampal neurons was significantly decreased in the NGR1 group, indicating that NGR1 can significantly decrease the ratio of apoptotic hippocampal neurons in rats with CIRI, and this effect is stronger than that of the positive control.

Effect of NGR1 treatment on hippocampal expression of BDNF mRNA. The relative levels of BDNF mRNA in the hippocampus of each group were measured by qRT-PCR. As shown in Fig. 3, compared with the Sham group, the expression of BDNF mRNA was significantly increased in the CIR group, while the expression of BDNF mRNA was significantly higher in the NGR1 and NDC groups compared with the CIR group. In addition, the expression of BDNF mRNA was significantly higher in the NGR1 group than in the NDC group. These data suggest that CIRI in rats can significantly increase the expression of BDNF mRNA, while NGR1 treatment can further increase the expression of BDNF mRNA in rats with CIRI, and this effect is stronger than that of the positive control.

Effects of NGR1 treatment on hippocampal Bcl-2 and Bax protein expression. Western blot analysis was used to measure the expression of Bcl-2 and Bax protein in the hippocampus in each group. As shown in Fig. 4, compared with the Sham group, the levels of the anti-apoptotic factor, Bcl-2, were significantly decreased, and the levels of the pro-apoptotic factor, Bax, were significantly increased in the CIR group, while the levels of Bcl-2 were significantly higher and those of Bax were significantly lower in the NGR1 and NDC groups compared with the CIR group. In addition, compared with the NDC group, the levels of Bcl-2 were significantly increased and those of Bax were significantly decreased in the NGR1 group compared with the NDC group. These data indicate that NGR1 treatment can significantly increase the expression of Bcl-2 protein and significantly decreased the expression of Bax protein in the hippocampus of rats with CIRI.

Discussion

Numerous studies have shown that oxidative stress plays an important role in the pathogenesis of acute ischemic stroke, and is an important cause of the ischemic injury cascade. In addition, oxidative stress can induce cellular necrosis and apoptosis (13). Studies have shown that cellular necrosis in the core part of the ischemic area can be induced in the transient state or within a few hours under oxidative stress, while cellular necrosis is slow in the semi-dark area and is mainly caused by Bcl-2 family-mediated apoptosis (14).

BDNF is a neurotrophic factor that is widely distributed in the cerebral cortex, hippocampus, and striatum. It has been

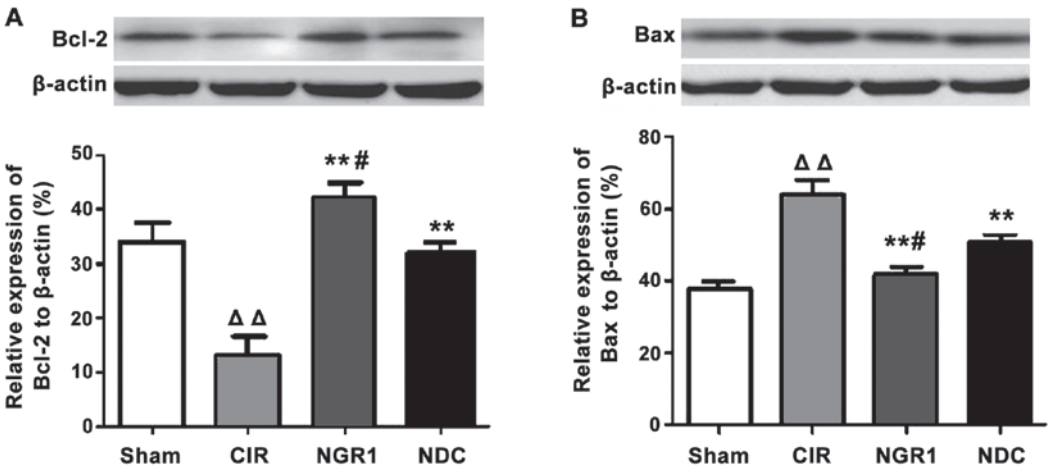

Figure 4. Hippocampal expression of Bcl-2 and Bax protein in each group at 24 h after surgery. Western blot analysis showed that the levels of the anti-apoptotic factor, Bcl-2, were significantly decreased and the levels of the pro-apoptotic factor, Bax, were significantly increased in the CIR group compared with the Sham group. The levels of Bcl-2 protein were significantly higher and the levels of Bax protein were significantly lower in the NGR1 and NDC groups compared with the CIR group. Compared with the NDC group, the levels of Bcl-2 protein were significantly increased and the levels of Bax protein were significantly decreased in the NGR1 group compared with the NDC group. $^{\Delta\Delta}$Compared with the Sham group, $p<0.01$; **compared with the CIR group, $p<0.01$; $^{\#}$compared with the NDC group, $p<0.05$. CIR, cerebral ischemia-reperfusion model group; NGR1, notoginsenoside R1 treatment group; NDC, nimodipine positive control group.

shown that BDNF plays an important role in protecting the patients from CIRI, and its high expression is beneficial for the improvement of pathologic conditions of the brain, and for the repair of neuronal damage in CIRI (15). The mechanism of this protection is complex, and is mainly achieved by stabilizing intracellular Ca^{2+} concentration balance by antagonizing the toxicity of excitatory amino acids, antagonizing cytotoxicity caused by nitric oxide, enhancing the activity of antioxidant enzymes, inhibiting the activity of caspase-3, and regulating the expression of Bcl-2 and Bax, which in turn reduces the occurrence of apoptosis and necrosis, and promotes the regeneration of damaged neurons (16).

Neurosurgical treatment targeting all aspects of cerebral ischemia or ischemia-reperfusion injury has received increasing attention. At present, neuroprotective drugs mainly include active oxygen free radical scavengers, anti-apoptotic agents, and anti-inflammatory agents, among which nimodipine is a commonly used drug in the treatment of cerebral ischemia. Nimodipine can improve blood circulation during the recovery of acute cerebrovascular disease and ischemic neurological disorders (such as hypertension and migraine) and cerebral vasospasm after subarachnoid hemorrhage caused by various factors. In addition, nimodipine can be used in the treatment of ischemic neuronal injury and vascular dementia. However, these chemicals can easily cause side effects, and drug tolerance can develop after long-term use, which in turn leads to unsatisfactory treatment outcomes. Numerous studies have shown that PNS is more efficient than neuroprotection in improving hypoxia tolerance, building immunity, anti-aging function, and other aspects (17). Monomeric saponins of PNS, such as NGR1, have shown satisfactory efficacy in the treatment of cerebrovascular, hematological, and neurological diseases, and for inflammation (18). Moreover, studies have shown that PNC can protect the activity of endogenous superoxide dismutase to reduce CIRI by removing free radicals produced by xanthine oxidase (19), and reduce the occurrence of neuronal apoptosis during ischemia-reperfusion by inhibiting the expression and activation of the key apoptotic protease, caspase-3. Other studies found that PNC can protect the brain by inhibiting the expression of Bax and reducing the rate of apoptosis of nerve cells after the occurrence of cerebral ischemia and hypoxia caused by asphyxia and reperfusion injury (20).

Our study found that NGR1 treatment in rats with CIRI significantly reduced the area of cerebral infarction, and significantly increased the expression of the BDNF gene in the hippocampus, which in turn protected hippocampal neurons from CIRI. This protective effect is achieved possibly by increasing the expression of the anti-apoptotic factor, Bcl-2, and synergistically reducing the expression of the Bcl-2 antagonist factor (Bax), which in turn regulates the expression of Bcl-2 family genes and inhibits neuronal cell apoptosis. This is consistent with the aforementioned findings. In addition, we found that the protective effects of NGR1 on rats with CIRI were stronger than those of the positive control, nimodipine. Therefore, NGR1 may represent a novel drug with promising prospects for the treatment of CIRI and other cerebrovascular diseases or acute ischemic stroke.

In conclusion, our study provided new insights into the treatment of CIRI, and a theoretical basis for the screening of drugs for the treatment of CIRI.

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