Role of the Janus kinase 2/signal transducers and activators of transcription 3 pathway in the protective effect of remote ischemia preconditioning against cerebral ischemia–reperfusion injury in rats

Yunlong Zhaoa,b,c, Yan Xuea,b,c, Zehan Liu a,b,c, Shuai Ren a,b,c, Xiangchen Guana,b,c, Ming Lia,b,c, Xin Zhaoa,b,c, Yang Songa,b,c and Xiaoping Ren a,b,c,d

Remote ischemia preconditioning (RIPC) is a convenient and effective method for alleviating cerebral ischemia–reperfusion injury (CIRI). However, to date, the underlying mechanism has not been fully elucidated. The aim of this research was to explore the protective mechanism of RIPC on the brain after CIRI. Four groups of rats were included in this experiment: the sham group, the middle cerebral artery occlusion (MCAO) group, the RIPC group, and the AG490 group. As an inhibitor of Janus kinase 2 (JAK2), AG490 was used after MCAO in the AG490 group to explore the role of JAK2/signal transducers and activators of transcription 3 (STAT3) after CIRI. Brain tissue was collected for evaluation after 2 h of ischemia and 24 h of reperfusion. ELISA for interleukin (IL)-6, IL-1β and tumor necrosis factor-α, western blot for phosphorylated-JAK2 and phosphorylated-STAT3, the neurological severity score and Longa scoring system for neurological deficit evaluation, triphenyltetrazolium chloride staining for cerebral infarction, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining for apoptotic cells in the brain tissue were performed. Neurological function in the RIPC group was notably better than that in the MCAO group. There were smaller infarction sizes and fewer apoptotic cells in the ischemic area in the RIPC group than in the MCAO group. In the RIPC group, the expression levels of IL-1β, tumor necrosis factor-α, IL-6, and phosphorylated-JAK2 and phosphorylated-STAT3 were significantly lower than those in the MCAO group. The findings in the RIPC and AG490 groups were similar. The inflammatory response and apoptosis are two important processes involved in brain dysfunction after CIRI. The JAK2/STAT3 signaling pathway has an underlying relationship with these two processes. These findings suggest that RIPC can alleviate the damage to brain tissue by CIRI by regulating the JAK2/STAT3 signaling pathway negatively.

Keywords: apoptosis, cerebral ischemia–reperfusion injury, inflammatory factor, Janus kinase 2/signal transducers and activators of transcription 3 signal pathway, remote ischemic preconditioning

Introduction

Brain dysfunction and hemiplegic paralysis are most often caused by stroke, which can lead to death in middle-aged and old-aged individuals worldwide [1]. Cerebral ischemia–reperfusion injury (CIRI) following stroke results in further exacerbation of brain damage and prevents nerve functional recovery [2]. For individuals with high stroke risk, such as a family history of stroke or hypertension and hyperlipidemia, stroke is induced easily after surgical anesthesia or acute trauma [3]. Therefore, finding effective methods to prevent CIRI has become a key area in treating cerebral ischemia. Ischemic preconditioning is one such method that consists of brief, repeated, nonlethal ischemic stimulation to the target organs [3]. Remote limb ischemia preconditioning (RIPC) is a variant of this technique, wherein a brief (noninjurious) ischemia in the vascular bed of the hindlimb protects the target organ (e.g. the brain) from subsequent ischemic injury [4]. The ischemic tolerance characteristics induced by RIPC are similar to the mechanisms of natural ischemic tolerance.

In the late stages of CIRI, the nerve cells in the peripheral penumbra region are in the apoptotic phase, and the final size of the cerebral infarction caused by CIRI is...
determined by the peripheral penumbra area [5]. As an important factor in the inflammatory response induced by CIRI, the activation of inflammatory factors including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 could enhance the inflammatory response and aggravate nerve damage [6,7]. The Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) pathway transfers the extracellular chemical signals to the nucleus and promotes the expression of proliferation-related, differentiation-related, immunity-related, and apoptosis-related genes [8,9]. However, the mechanism underlying the protective effect of RIPC on CIRI is still uncertain. We used AG490, a specificity inhibitor of JAK2, to clarify the effect of the JAK2/STAT3 signaling pathway in CIRI and applied RIPC to a middle cerebral artery occlusion (MCAO) model to study the therapeutic effects. Here, we have expanded the extant literature on stroke and hypothesized that the JAK2/STAT3 signaling pathway plays a role in the protective effects of RIPC.

Materials and methods
The experimental protocol was in line with the requirements of the Institute of Laboratory Animal Science of China (AS655-01) and the Directive 2010/63/EU of the European Parliament on the guidelines of animal research.

Animals and grouping
Male 260–280 g, 7–8-week-old Sprague-Dawley rats were supplied by the Experiment Animal Center of the Second Affiliated Hospital of Harbin Medical University. The animals were housed at a temperature of 20–22°C with a 12 h alternating light–dark cycle under standard laboratory conditions and were allowed free access to water and food. There were four groups of rats: the sham group, the MCAO group, the RIPC group, and the JAK2 inhibitor (AG490) group. Each group included 40 rats and was included in five tests, with eight rats used for each test. A total of 165 rats, including two animals that died and three animals excluded postoperatively (failure in the establishment of MCAO model), were used. Thoracotomy for the rats in all groups was performed similarly, but the bilateral hindlimbs were not tied by gauze ropes. The animals were returned to their cages after RIPC and allowed access to food and water. The RIPC operation was performed 24 h before the establishment of the MCAO model.

The establishment of the middle cerebral artery occlusion model
The MCAO model was adopted to induce transient focal cerebral ischemia. Animals were injected with 1% pentobarbital sodium salt (90 mg/kg) intraperitoneally and fixed in the supine position. A median incision (2–3 cm) was made on the neck to expose the right common carotid artery, internal carotid artery, and external carotid artery. A small v-shaped incision was made at the distal end of the right common carotid artery by ophthalmic clamping. The embolus line was inserted into the small v-shaped incision. Along the internal carotid artery, the embolus line was inserted into the initial segment of the middle cerebral artery. The respiration rate of the rats was maintained at 70–110 breaths/min. The body temperature of rats was maintained at 37±0.5°C intraoperatively by a constant temperature apparatus. During MCAO, the reduction of unilateral cerebral blood flow was more than 70% compared with the baseline levels, as measured by Laser Doppler flowmetry (PeriFlux 5000; Perimed, Beijing, China). The entire MCAO process lasted 2 h. Following MCAO, the embolus line was removed for reperfusion. Three groups of rats were included in this process: the MCAO, RIPC, and AG490 groups. The rats in the sham group were anesthetized and exposed to right MCA without occlusion.

The application of AG490
AG490 is an inhibitor that may prevent the phosphorylation of JAK2 and downregulate the levels of pJAK2 and pSTAT3 in the brain. By comparing the AG490 group with the MCAO group, we investigated the role of the JAK2/STAT3 signaling pathway in CIRI. AG490 (40 mg/kg dissolved in 3% DMSO) was injected intraperitoneally 5 min before reperfusion in the AG490 group [11]. All other groups received 3% DMSO as a control. All tests were conducted after 24 h of reperfusion.

Neurological deficit evaluation
We evaluated neurological deficits 24 h after reperfusion. The observers were blinded to the experimental groups. Scoring was performed according to the neurological severity score (Table 1) and the Longa scoring system (0: no dysfunction; 1: failure to fully extend the contralateral forelimb when the tail is lifted; 2: tendency to...
Table 1 Neurological severity scores

| Tests                              | Points |
|-----------------------------------|--------|
| Motor tests                        | 6      |
| Placing rat on the floor           | 3      |
| Normal walk                        | 0      |
| Inability to walk straight         | 1      |
| Circling toward the paretic side   | 2      |
| Fall down to the paretic side      | 3      |
| Raising rat by the tail            | 3      |
| Flexion of forelimb                | 1      |
| Flexion of hindlimb                | 1      |
| Head moved > 10° to vertical axis within 30 s | 1 |
| Beam balance tests                 | 6      |
| Balances with steady posture       | 0      |
| Grasps side of beam                | 1      |
| Hugs the beam and one limb falls down from the beam | 2 |
| Hugs the beam and two limb falls down from the beam, or spins on beam (>60 s) | 3 |
| Attempt to balance on the beam but fall off (>40 s) | 4 |
| Attempt to balance on the beam but fall off (>20 s) | 5 |
| Falls off: no attempt to balance or hang on the beam (<20 s) | 6 |
| Sensory tests                      | 2      |
| Placing test: visual and tactile test | 1 |
| Proprioceptive test: deep sensation, pushing the paw against the table edge to stimulate limb muscles | 2 |
| Reflexes absent and abnormal movements | 4 |
| Pinna reflex: head shake when touching the auditory meatus | 1 |
| Corneal reflex: eye blink when lightly touching the cornea with cotton | 1 |
| Startle reflex: motor response to a brief noise from snapping a clipboard paper | 1 |
| Seizures, myoclonus, mydystony     | 1      |
| Total points                       | 14     |

0: no function damage; 1–6: mild damage; 7–12: moderate damage; 13–18: severe damage.

spin in the direction of the paralyzed side; 3: tendency to lean toward the paralyzed side; 4: inability to walk and disruption of consciousness; and 5: death) [12]. A score of 1–3 points in the animals indicated success in the establishment of the MCAO model. Animals were assessed 24 h after surgery.

Measurement of cerebral infarct size
We used TTC to measure the size of the cerebral infarction in the rats [13]. According to the manufacturer's instructions, brain tissue was sectioned and stained. The infarct volume was calculated as the area of the contralateral hemisphere minus the area without edema on the ipsilateral hemisphere.

Apoptotic cell analysis
According to the protocol for the in-situ Brdu-Red DNA Fragmentation Assay Kit, the brain tissue sections were stained with TUNEL and DAPI. The cells were analyzed by fluorescence microscopy. TUNEL-positive cells with blue-stained apoptotic bodies were considered apoptotic cells [10]. An examiner blinded to the experimental groups selected six high-power regions in the infarct area of each rat brain slice for evaluation under a microscope at a magnification of ×200. We used imaging software to count the total number of cells and the number of apoptotic cells in each field of view.

Cytokine assay
As described previously, ELISA kits (Abcam, Shanghai, China) were used to measure the concentrations of TNF-α, IL-1β, and IL-6 in the brain tissue of the rats after CIRI for 24 h. A microplate reader was used to measure the absorbance and draw the histogram.

Western blot analysis
Western blotting was used to detect changes in the levels of pJAK2 and pSTAT3. The process was performed in strict accordance with the manufacturer's instructions.

Statistical analysis
Statistical analyses were carried out using SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA) statistical software. Multiple-group comparisons were made by one-way analysis of variance, followed by Tukey’s test. Student’s t-test was used for comparison between two groups. The results were expressed as the mean ± SEM. A value of P less than 0.05 was considered statistically significant.

Results
Remote ischemia preconditioning improves neurological deficits caused by ischemic-reperfusion injury
The results of the modified neurological severity score and the Longa scoring system are shown in Fig. 1. There was no neurological impairment in sham rats. MCAO rats scored significantly higher on the two scoring systems than rats in the other groups. Rats in the RIPC group and the AG490 group had lower scores than the rats in the MCAO group (P < 0.05). For all groups, the trends observed in the data from the two scoring systems were essentially identical.

Remote ischemia preconditioning reduces brain infarction size
TTC staining was used to examine the infarction size in the rats from each group after CIRI. In the sham group, no infarction was observed. The results from the MCAO group showed that CIRI increased the infarction size, which was decreased by RIPC and AG490 (P < 0.05; Fig. 2).

Remote ischemia preconditioning reduces cerebral ischemia–reperfusion injury-induced neuronal apoptosis
Compared with the sham group, the MCAO group showed a significant increase in TUNEL staining. This result suggested a higher number of apoptotic neuronal cells (P < 0.05), whereas the percentage of neuronal cells that were apoptotic in the RIPC group and AG490 group was less than that in the MCAO group (P < 0.05; Fig. 3).

Remote ischemia preconditioning relieves the cerebral ischemia–reperfusion injury-induced inflammatory response
We observed that TNF-α, IL-1β, and IL-6 were maintained at normal levels in the sham group and were
clearly increased in the MCAO group ($P < 0.05$). However, compared with those in the MCAO group, the levels of inflammatory cytokines in the RIPC group and AG490 group declined markedly ($P < 0.05$; Fig. 4).

Remote ischemia preconditioning reduces the expression of phosphorylated Janus kinase 2 and phosphorylated signal transducers and activators of transcription 3 after cerebral ischemia–reperfusion injury

The expression levels of pJAK2 and pSTAT3 were the lowest in the sham group and the highest in the MCAO group ($P < 0.05$), whereas RIPC and AG490 significantly reduced the increase in pJAK2 and pSTAT3 induced by CIRI ($P < 0.05$; Fig. 5).

Discussion

The protective function of RIPC in CIRI has been recognized widely [14]. In this experiment, we used an MCAO model to simulate the process of focal CIRI and verified the effects of RIPC on cerebral CIRI to explore its mechanism of action. Previous studies have shown that CIRI is associated with inflammatory responses and nerve damage. In this study, we showed that the JAK2/STAT3 signaling pathway was a significant factor in the protective role of RIPC after CIRI using AG490 and found that RIPC reduced not only neurological dysfunction but also the inflammatory response and nerve cell apoptosis in an MCAO rat model. The application of RIPC can effectively improve the survival rate and prognosis after a sudden stroke. This experiment provides a theoretical basis for clinical applications.

The pathogenesis of ischemic stroke is very complicated. Previous experiments have shown that the inflammatory response plays a huge role in the prognosis of neurological function after CIRI [15]. Following CIRI, the accumulation of inflammatory mediators, the activation and infiltration of inflammatory cells, and the destruction of the blood brain barrier eventually lead to the aggravation of inflammation and a series of serious pathophysiological changes that result in brain damage [16]. As an important factor in inflammation after CIRI, the
activation of TNF-α, IL-1β, and IL-6 could aggravate the inflammatory response [17]. According to a study, peripheral macrophages activate the JAK2/STAT3 pathway, promoting the release of TNF-α. Another study showed that rapamycin downregulates the JAK2/STAT3 signaling pathway and reduces inflammation caused by liver injury [18]. In a study carried out on the intestines, the results showed that the upregulation of pJAK2 and pSTAT3 induces inflammation, which could be decreased by AG490 [19]. These studies suggest an intrinsic link between the JAK2/STAT3 signaling pathway and the inflammatory response, suggesting that the
inflammatory response rapidly activates the JAK/STAT signaling pathway. Interactions between IL-6 and its corresponding receptors after CIRI cause the phosphorylation of JAK, STAT3 and dimers of pSTAT3, which are then transferred to the cell nucleus. The combination of dimers and DNA leads to increased expression of cytokines such as interleukins, generating more TNF-α, IL-1β, and IL-6. This cycle enhances the inflammatory response and aggravates nerve damage [20]. In this experiment, we proved that RIPC could reduce the generation of TNF-α, IL-1β, and IL-6, thus alleviating the severity of the inflammatory response by ELISA. The results suggest that RIPC can effectively block the IL-6 inflammatory response cycle and reduce the expression of IL-6 in the JAK2/STAT3 signaling pathway, thereby reducing the neurological damage caused by ischemic inflammation.

Neuronal apoptosis is considered to be another important pathological process in CIRI [21]. Apoptotic cells are mainly concentrated in the peripheral penumbra of cerebral infarction and are closely related to the prognosis of neurological function and decreasing the infarction size. This process of apoptosis is reversible. Previous studies have shown that negative regulation of the JAK2/STAT3 pathway had a positive effect on the reduction of cerebral infarction [22]. According to a study, miR-24 can reduce neuronal apoptosis, whereas the downregulation of JAK2/STAT3 leads to the upregulation of miR-24 [23]. In this study, we verified that RIPC could reduce the area of cerebral infarction and the impairment of neurological function after CIRI. Through western blotting, we showed that RIPC could reduce the expression of pJAK2 and pSTAT3. The results suggest that RIPC can effectively downregulate the JAK2/STAT3 signaling pathway by inhibiting the phosphorylation of JAK2, accelerating the recovery of nerve cells during apoptosis, reducing the number of apoptotic cells, eventually reducing the cerebral infarction size and improving neurological function.

In this experiment, we compared the protective effects on the brain after CIRI in the RIPC group with those in the AG490 group. In each experiment, the data from the two groups were relatively similar and showed no statistical significance (P<0.05). This finding confirms that the regulatory effect of RIPC on the JAK2/STAT3 pathway is similar to that of AG490. The transduction process of the JAK2/STAT3 signal pathway involves many steps, beginning with the binding of cytokine receptors to their related ligands to activate JAK2. pJAK2 then activates and phosphorylates STAT3. Finally, pSTAT3 is translocated to the nucleus and combines with a specific regulatory sequence on the DNA to regulate the transcription of genes [24,25]. As an inhibitor, AG490 blocks the phosphorylation of JAK2, thereby reducing the expression of all the proteins involved in the JAK2/STAT3 pathway.
JAK2/STAT3 pathway. In the present research, we revealed by western blotting that RIPC can reduce the levels of pJAK2 and pSTAT3, showing that RIPC also blocks the phosphorylation of JAK2. Whether RIPC can block the phosphorylation of STAT3 concurrently needs to be further investigated.

Conclusion
RIPC prevents the phosphorylation of JAK2, thereby reducing the expression of pSTAT3 in the ischemic infarct area, apoptosis, the inflammatory response, and the size of the cerebellar infarction and improving neurological function. RIPC provides a new and effective method for the treatment of cerebral local transient ischemic diseases.

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Conflicts of interest
There are no conflicts of interest.

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