ORIGINAL ARTICLE

AKT/GSK3 β -Dependent Autophagy Contributes to the Neuroprotection of Limb Remote Ischemic Postconditioning in the Transient Cerebral Ischemic Rat Model

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Keywords

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SUMMARY

Background: Limb remote ischemic postconditioning (RIPostC) has been recognized as an applicable strategy in protecting against cerebral ischemic injury. However, the time window for application of limb RPostC and the mechanisms behind RPostC are still unclear. **Aims:** In this study, we investigated the protective efficacy and the role of autophagy in limb RPostC using a transient middle cerebral artery occlusion rat model. **Results:** Limb RPostC applied in the early phase of reperfusion reduced infarct size and improved neurological function. Autophagy levels in penumbral tissues were elevated in neurons of limb RPostC rats, with an increase in the phosphorylation of AKT and glycogen synthase kinase 3 β (GSK3 β). Blocking the AKT/GSK3 β pathway via the AKT inhibitor LY294002 prior to limb RPostC suppressed the RPostC-induced autophagy and resulted in the activation of caspase-3 in RPostC rats, suggesting a critical role for AKT/GSK3 β -dependent autophagy in reducing cell death after cerebral ischemia. **Conclusions:** These results aid optimization of the time window for RPostC use and offer novel insight into, and a better understanding of, the protective mechanism of autophagy in limb RPostC.

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The first two authors contributed equally to this work.

Introduction

Stroke is an acute disease with serious complications and high mortality that is caused by a disturbance in the blood supply to the brain. It is critical to take effective measures as early as possible after stroke occurrence to reduce ischemic injury and protect neuronal functions. To this end, ischemic postconditioning has recently been reported to be a promising approach [1–3].

Limb remote ischemic postconditioning (RIPostC) is a newly developed postconditioning procedure that involves repeated occlusion/release cycles in the bilateral femoral arteries. Unlike the classical pre- or postischemic conditioning, limb RPostC is adaptable in practice. More and more studies indicate that limb RPostC improves outcome in ischemic animal models [4–6]. Our previous study also suggested that limb RPostC reduces brain edema and blood–brain barrier disruption in experimental ischemia/reperfusion in rats [7]. However, the time window and

the protective mechanism for limb RPostC have not been well investigated in the transient middle cerebral artery occlusion (MCAO) animal model.

Autophagy is a vital cellular pathway for the degradation of intracellular macromolecules or organelles for subsequent reuse that helps to maintain intracellular homeostasis in physiological conditions. Recent studies suggested that autophagy is an important arbiter of cell death-survival decisions by degrading these harmful aggregates and organelles in inflammation [8], malignancy [9], and neurodegeneration [10]. Autophagy is also reported to be involved in cerebral ischemia [11,12], but its role in the associated neuronal death is still controversial. Some reports have indicated that autophagy promoted apoptosis in primary cortical neurons [13] or in animal models of cerebral ischemia [14]. However, other studies suggested that induction of autophagy contributed to the neuroprotection afforded by nicotinamide phosphoribosyltransferase [15] and ischemic preconditioning [11].

In this study, we aimed to clarify the role of autophagy in neuronal cell death/survival and to investigate the time window and possible mechanism of autophagy induction during limb RPostC application after transient ischemia in the MCAO rat model.

Materials and Methods

Animal Model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University, Beijing, and were in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley rats (300–320 g in weight) were anesthetized with 1.75% isoflurane in 70% nitrous oxide and 30% oxygen. For the MCAO model, ischemia and reperfusion (I/R) was established as described previously [16]. Briefly, the right external carotid artery (ECA), internal carotid artery (ICA), and pterygopalatine artery of the ICA were exposed. Occlusion was conducted by inserting a nylon suture with a diameter of 0.38 mm into the ICA via a slit on the ECA. The suture was advanced along the ICA to 18–19 mm from the bifurcation and maintained in position for 2 h. Reperfusion was produced by gently withdrawing the suture until the suture tip reached the bifurcation. Successful MCAO and reperfusion was confirmed by laser Doppler flowmetry (PeriFlux System 5000; Perimed, Stockholm, Sweden). Rats that showed convulsions or sustained consciousness disturbances were excluded from the subsequent experiments, as most of these animals were identified to have suffered subarachnoid hemorrhage secondary to suture-induced rupture of the ICA. The animals in the sham group underwent the identical procedure except occlusion was omitted.

In the I/R + RPostC groups, limb RPostC was carried out by three cycles of 10 min occlusion/10 min release of the bilateral femoral artery using clamps at 0, 10, and 30 min of reperfusion (R-0, R-10, R-30 groups, respectively). The protocol for limb RPostC in each group is schematically illustrated in Figure 1A. Except for the experiment on cerebral infarction and neurological function, all studies were performed in animals of the sham, I/R and I/R + R-0 groups.

To address the role of AKT, the AKT antagonist LY294002 (30 μ M in 10 μ L; Sigma, St. Louis, MO, USA) or DMSO (vehicle control) was microinjected into the lateral ventricle 30 min prior to MCAO surgery. Stereotaxic coordinates for injection were bregma posterior (P) -1 mm; lateral (L) ± 1.5 mm; and ventral (V) -4.0 mm from the dura, with the tooth bar set at 0 mm.

Measurement of Neurological Deficits

Neurological deficits were assessed at 22 h after reperfusion in a double-blind fashion using the method of Ludmila Belayev [17] and foot-fault tests [18]. For the Ludmila Belayev score, neurological function was graded on a scale of 0–12, with 0 representing normal function and 12 representing maximum neurological deficits. The score was derived from two tests: (1) the postural reflex test to examine upper body posture while rats were suspended by

Figure 1 Depending on the time of application, Limb RPostC reduces infarct volumes. (A) The schematic protocol for limb RPostC. I/R rats (ischemia for 2 h and reperfusion for 22 h) that underwent three cycles of 10-min occlusion (yellow rectangles)/10-min release of bilateral femoral artery during reperfusion are indicated as limb RPostC treatment. According to the start of conditioning (0, 10 or 30 min after reperfusion), RPostC rats were divided into three groups: I/R + R-0, I/R + R-10, and I/R + R-30. (B) Representative brain slices stained by TTC in I/R and the three RPostC groups. (C) Quantitative evaluation of infarct volumes in different groups. Data are presented as mean $\pm$ SEM from eight animals. * $P < 0.05$, ** $P < 0.01$ versus the I/R group.

the tail, and (2) the forelimb placing test to examine sensory motor integration in forelimb placing in response to visual, tactile, and proprioceptive stimuli. For the foot-fault tests, rats were placed on and traversed along a 110-cm-length platform with $9 \times 9 \text{ cm}^2$ grids. The number of times the forelimb fell into the grids within 1 min was counted to reflect neurological function deficits.

Infarct Volume by TTC Staining

The infarct volume was measured at 22 h of reperfusion by 5-triphenyl-2H-tetrazolium chloride (TTC; Sigma) staining as described previously [19]. Briefly, rats were transcardially perfused with phosphate-buffered saline under deep anesthesia to eliminate intravascular blood. Brains were quickly removed and sectioned into six 2-mm-thick consecutive coronal slices and then stained with 1.5% TTC. The infarct volume of each brain was measured using Image-ProPlus Analysis Software (Media Cybernetics, Inc., Rockville, MD, USA).

SDS-PAGE and Western Blotting Analysis

Twenty-two hours after reperfusion, rats were transcardially perfused with PBS and the penumbral tissues were collected and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1 mM Na_2EDTA , 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 , and 1 $\mu\text{g/mL}$ leupeptin). Homogenates were sonicated and centrifuged at 18,000 *g* for 30 min at 4°C. The protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Pierce, Biotechnology, Rockford, IL, USA). SDS-PAGE and Western blotting were carried out as previously described [20]. Primary antibodies used for immunoblotting were as follows: anti-p-S473-AKT (1:1000), anti-p-S9-GSK3 β (1:1000), anti-LC3 (1:1000), anti-AKT (1:1000), anti-GSK3 β (1:1000), and anti- β -actin (1:1000), all of which were from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated secondary antibodies (1:5000) were from Chemicon (Billerica, MA, USA). The expression of β -actin in the same membrane was simultaneously determined as an internal reference. Signals were visualized using the LumiGLO chemiluminescent substrate, and immunoblots were quantitatively analyzed after scanning of the X-ray films using AlphaEaseFC software (Alpha Innotech, San Francisco, CA, USA).

Immunohistochemistry Staining

Immunostaining was carried out at 22 h of reperfusion. Rats were transcardially perfused with PBS, followed by 4% paraformaldehyde. Twenty-micron-thick cryostat sections were obtained and double-stained with rabbit anti-p-S9-GSK3 β (1:100; Cell Signaling Technology), anti-LC3 (1:150; Cell Signaling Technology), and mouse anti-Neuron-Specific Nuclear Protein (NeuN, 1:50; Millipore, Billerica, MA, USA) or antigenial fibrillary acidic protein (GFAP, 1:50; Millipore). Five-micron-thick paraffin-embedded slices were stained with anticlaved caspase-3 (1:50; Cell Signaling Technology) and counterstained with DAPI. Images were captured using a microscope (Nikon, Tokyo, Japan).

Assessment of Autophagy Level

Microtubule-associated protein light chain-3 (LC3) undergoes successive modification to form LC3-I and phosphatidylethanolamine-conjugated LC3-II that is specifically targeted to autophagosome membranes [21]. To evaluate the autophagy level, two groups of experiments were conducted. First, the LC3-II/LC3-I ratio was determined in each condition by Western blotting and expressed as the percentage of that of the sham group. Next, the number of LC3-positive puncta was assessed by immunostaining as a measure of the autophagy level. For this purpose, paraffin-embedded slices were double-stained with anti-LC3 (1:50; Cell Signaling Technology) and mouse anti-NeuN (1:50; Millipore). LC3-positive puncta in penumbral tissues were counted in each group.

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's *post hoc* correction for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

The Protective Time Window of Limb RIPOstC Against Transient Cerebral Ischemic Injury

To ascertain the time window for limb RIPOstC in protection against I/R injury in the MCAO rat, limb RIPOstC was carried out within different time periods during reperfusion. The outcome was first assessed by examination of cerebral infarction using TTC-stained brain slices from the different experimental groups (Figure 1B). In comparison with the I/R group, the infarct volume in all RIPOstC groups but the R-30 groups was significantly reduced with a quantitative analysis of infarct volume showing a significant difference between the I/R ($48\% \pm 3.3$) and I/R + RIPOstC groups (R-0: $26\% \pm 4.4$; R-10: $30\% \pm 3.5$), with the exception of R-30 ($40\% \pm 3.1$; $n = 8$; Figure 1C).

The effect of RIPOstC after different time periods was evaluated further using the Ludmila Belayev neurological score and foot-fault tests at the end of 22 h of reperfusion. Although Ludmila Belayev's neurological scores (I/R: 7.25 ± 0.25 ; I/R + R-0: 6.62 ± 0.63 ; I/R + R-10: 6.62 ± 0.50 ; I/R + R-30: 8.00 ± 0.27) did not show a statistically significant difference between the groups ($n = 8$; Figure 2A), the result of the foot-faults test revealed that RIPOstC in the R-0 and R-10 groups significantly improved neurological function when compared to the I/R group (I/R: 8.12 ± 0.58 ; I/R + R-0: 6.00 ± 0.80 ; I/R + R-10: 5.25 ± 0.75 ; I/R + R-30: 7.75 ± 0.37 ; $n = 8$; Figure 2B), indicating that MCAO rats benefit more from RIPOstC conducted in the early phase of reperfusion.

Limb RIPOstC Promotes Autophagy in NeuN-Positive Cells in the Penumbra

To explore the mechanism of action of RIPOstC, we focused on the I/R + R-0 group because the animals in this group exhibited

Figure 2 Depending on the time of application Limb, RI-PostC improves neurological function. **(A)** Ludmila Belayev's neurological scores. Rats from the three RI-PostC groups did not significantly differ from the I/R group with regard to neurological function. **(B)** Foot-faults test. The rats in the I/R + R-0 and I/R + R-10 groups showed significantly improved neurological function in comparison with the I/R group. The data are presented as mean ± SEM from eight animals. * $P < 0.05$ versus the I/R group.

significantly less I/R injury in comparison with the I/R group, as evidenced by both reduced infarct volume and improved neurological function. The level of autophagy in the penumbra at 22-h reperfusion was assessed by Western blotting of LC3 (Figure 3A). Normalizing the statistic data with the sham group (Figure 3B) revealed a significant increase in the LC3-II/LC3-I ratio in the I/R + R-0 group ($162.01\% \pm 30.49$) compared with I/R rats ($112.65\% \pm 16.01$; $n = 4$; Figure 3B). To identify the cell type where the autophagy was occurring, penumbral tissue was double immunostained with antibodies for LC3 and either neuron-specific NeuN or astrocyte-specific GFAP (Figure 3C). The results demonstrated that autophagy took place mostly, if not exclusively, in neurons (images a, b, c), but not in astrocytes (images e,

f, g). The images in the dashed-line rectangles in (c) and (g) were magnified and shown in (d) and (h).

AKT/GSK3 β Phosphorylation is Involved in RI-PostC-Induced Autophagy

As the AKT/GSK3 β pathway has been proposed to be an important protective mechanism in cerebral ischemia, we investigated whether limb RI-PostC leads to AKT/GSK3 β activation. For this purpose, the levels of S473-phosphorylated AKT (p-AKT) and S9-phosphorylated GSK3 β (p-GSK3 β) in the penumbra were studied at 22-h reperfusion by Western blotting (Figure 4A,C). Quantitative evaluation demonstrated that the expression of p-AKT in the penumbra significantly increased in I/R + R-0 rats when compared to I/R group (I/R: $103.17\% \pm 9.86$; I/R + R-0: $160.00\% \pm 17.94$; $n = 4$; Figure 4B). Likewise, a significant increase in the expression of p-GSK3 β was observed in the RI-PostC group when compared with I/R rats (I/R: $87.08\% \pm 19.92$; I/R + R-0: $149.60\% \pm 24.68$; $n = 4$; Figure 4D).

To address the role of the AKT/GSK3 β pathway in RI-PostC-induced autophagy, LY294002, an antagonist of AKT, was microinjected into the lateral ventricle 30 min before ischemia induction. The extent of the transformation of LC3 into LC3-I and -II and the levels of phosphorylated GSK3 β were determined by Western blotting at 22 h of reperfusion (Figure 5A). Analysis revealed that the level of autophagy reduced to $61\% \pm 13.26$ in comparison with that of the DMSO control group (Figure 5B; $n = 4$). This result was verified by an immunohistochemistry study that showed that both the number of NeuN-positive cells in the LY294002-treated group (d) and the level of phosphorylated GSK3 β (e) were reduced in comparison with the DMSO control (a, b; Figure 5C). Thus, inhibiting AKT activity significantly reduced both RI-PostC-induced autophagy and the expression of p-GSK3 β , suggesting that the AKT/GSK3 β pathway may play a critical role in RI-PostC-induced autophagy.

Suppressing Autophagy by AKT Inhibitor Activates Caspase-3 in RI-PostC Rats

To address the role of AKT/GSK3 β pathway in mediating cell survival in I/R injury and RI-PostC treatment, the expression of cleaved (activated) proapoptotic caspase-3 was assessed in the penumbra by immunohistochemistry at 22 h of reperfusion (Figure 6A). Caspase-3-positive cells were much more common in the penumbra (b) than in contralateral tissue (a). The number of caspase-3-positive cells declined, however, in the RI-PostC group (c), showing the beneficial role of RI-PostC in attenuating I/R injury. Moreover, the inhibitory effect of RI-PostC on caspase-3 expression was reversed by pretreatment with LY294002, suggesting implication of the AKT/GSK3 β pathway in mediating the effect of RI-PostC (d). The levels of cleaved caspase-3 were further verified by Western blotting (Figure 6B). Normalizing the data with those of the sham group showed that limb RI-PostC significantly suppressed I/R-induced caspase-3 activation, while LY294002 activated caspase-3 by blocking AKT/GSK3 β -dependant autophagy (I/R: $163.27\% \pm 20.57$; I/R + R-0: $122.10\% \pm 8.21$; I/R + R-0 + LY294002: $160.83\% \pm 8.36$; $n = 4$; Figure 6C).

Figure 3 Limb RPostC promotes autophagy in NeuN-positive cells of the penumbra. The autophagy level was measured in the I/R + R-0 group after 22-h reperfusion. **(A)** Western blotting for LC3 in penumbral tissue. **(B)** Data demonstrating that the level of autophagy, with the LC3-II/LC3-I ratio as an index, significantly increased in I/R + R-0 group when compared with I/R rats. Data are presented as mean $\pm$ SEM from four animals. * P < 0.05 versus the I/R group. **(C)** Immunohistochemistry of penumbral tissue of rats in the I/R + R-0 group after 22-h reperfusion by double-staining with LC3 (red) and neuron-specific NeuN or astrocyte-specific GFAP (green). LC3-positive cells (arrow head) were largely colocalized with NeuN-positive cells (**a**, **b**, **c**), not with astrocytes (**e**, **f**, **g**). The images in the dashed-line rectangles are magnified in **(d)** and **(h)**. Scale bar = 10 μ m.

Discussion

Ischemic conditioning, including both pre- and postconditioning, is regarded to be an efficacious protective strategy against brain ischemic injury and is thus attracting extensive interest [7,22–24]. Limb RPostC, which differs from conventional postconditioning

as it involves repetitive reperfusion and occlusion of bilateral femoral arteries, has been suggested to be a neuroprotective strategy that is easier to translate into clinical situations than RPostC of the common carotid artery (CCA) or other remote organs [25]. Recent studies and our previous results indicate that limb RPostC may reduce infarct size, brain edema, blood–brain barrier

Figure 4 AKT and GSK3 β are phosphorylated in RPostC-induced autophagy after cerebral ischemia. Western blotting assessment of AKT/GSK3 β phosphorylation in the penumbra was performed in the I/R + R-0 group, as well as in the I/R and sham groups, after 22-h reperfusion to detect S473-phosphorylated AKT (**A**) and S9-phosphorylated GSK3 β (**C**), with quantification of the results shown in (**B**) and (**D**). Data are expressed as mean $\pm$ SEM from four animals. * P < 0.05 versus the I/R group.

disruption and improve neurologic functions in stroke animals [7,23,26].

In this study, we observed that limb RPostC conducted within 10 min of reperfusion exhibited an unambiguous beneficial effect, as evidenced by both reduced infarct size and improved neurological function after 2 h of ischemia and 22 h of reperfusion. Our findings suggest that the best outcome may be expected if limb RPostC is applied in the early phase of reperfusion (Figures 1 and 2). A study of Sun et al. [23] demonstrated the neuroprotection of limb RPostC (performed 3 or 6 h after reperfusion) against 90-min ischemia followed by 72-h reperfusion, which corroborates our findings. However, it is necessary to investigate the long-term protective efficacy of limb RPostC in further experiments.

Autophagy, an important cell clearance system for degrading wastes or impaired organelles into micromolecules for reuse, has been reported to play an important role in ischemia [15,27]. In this study, we investigated whether autophagy was involved in the neuroprotection of RPostC after cerebral ischemia. Our results demonstrated for the first time that autophagy is promoted in the penumbra of cerebral ischemic rats with RPostC application (Figure 3), which is supported by the study of Park et al. [28] of ischemic preconditioning in PC12 cells following oxygen-glucose deprivation. Furthermore, our results showed that RPostC-induced autophagy is triggered mostly in neurons (NeuN-specific cells) of the penumbra and not in GFAP-positive astrocytes, which is supported by Tian's report [12] that used a live green fluorescent protein (GFP)-fused LC3 transgenic mouse model to show that after 60-min transient cerebral ischemia the GFP-LC3-positive cells of the peri-ischemic area were primarily neuronal, not

astro- or microglial. Tian's report also showed that autophagy was activated with a peak at 1 day, which was not consistent with our finding that a significant increase in autophagy was not seen in I/R rats that underwent 2-h ischemia and 22-h reperfusion. The main reason for this discrepancy may be the variation between the tissues that were collected for analysis, which may show a difference in the relative numbers of neurons and glia. Our studies suggest that autophagy may be critically important in limb RPostC, especially in the regulation of neuronal function and that autophagy may thus be a potential target for neuroprotective therapy after cerebral ischemia.

The mechanism of autophagy induction by limb RPostC application in MCAO rats was also explored. AKT/GSK3 β pathway has been recognized as a protective pathway against cerebral ischemic injury [29,30]. Recent studies also provided evidence for the involvement of AKT in autophagy induction by targeting key molecules in autophagy pathway [31,32], such as mammalian target of rapamycin (mTOR) [33] and phosphatidylinositol-3 kinase (PI3K) [34]. In this study, we demonstrate that limb RPostC upregulated the phosphorylation of AKT and GSK3 β at 22 h of reperfusion. Moreover, LY294002, an inhibitor of the AKT pathway, blocked RPostC-induced autophagosome formation by inhibiting GSK3 β phosphorylation, suggesting that AKT/GSK3 β phosphorylation plays a critical role in the regulation of RPostC-promoted autophagy. Our results are consistent with Peng's report [4], which found that RPostC of the hind limb upregulates the phosphatidylinositol-3 kinase/AKT (PI3K/AKT) pathway in the CA1 region in global cerebral I/R rats. Gao et al. [30] also reported that the AKT signaling pathway contributes to postconditioning's protection

Figure 5 LY294002 suppresses RPostC-induced autophagy after cerebral ischemia. To further characterize the role of AKT/GSK3 β pathway in RPostC-induced autophagy, the AKT inhibitor LY294002 was microinjected into the lateral ventricle of rats of the I/R+R-0 group 30 min before ischemia. **(A)** Expression of LC3 and p-GSK3 β was studied by Western blotting after 22-h reperfusion. **(B)** Statistical data showing that the level of autophagy was significantly suppressed in LY294002-injected rats when compared to I/R rats. Data are presented as mean $\pm$ SEM from four animals. * P < 0.05 versus the I/R group. **(C)** The number of NeuN-positive cells (green) was shown to mark reduced numbers of neurons (arrow head) in LY294002 **(d)** and vehicle control **(a)** groups. GSK3 β phosphorylation (red) was also shown in **(e)** and **(f)**. Scale bar = 10 μ m.

against stroke. Our and other findings suggest the critical role of AKT in autophagy promotion in ischemia conditioning.

Although autophagy is well known as being involved in type II cell death, the protective role of autophagy has been shown in many recent studies in ischemia [11,15,35]. In this study, we investigated the role of autophagy in cell death/survival during RPostC after stroke, particularly in penumbral tissues. By assessment of activated caspase-3, it was shown that I/R-induced apoptosis is attenuated by limb RPostC, but worsened by suppressing autophagy using the AKT inhibitor LY294002. This result suggests that autophagy may be an important protective mechanism in limb RPostC. Although our results suggested

that autophagy may be involved in the neuroprotection of limb RPostC, how autophagy reduces ischemic injury is still unclear. It is likely that autophagy promoted by RPostC application at the early phase of I/R injury protects neurons by accelerating the clearance of wastes or harmful signals and thereby blocking the neurotoxic cascade, for example, with upregulation of Bcl-2 and heat-shock protein 70 or reductions in cytochrome *c* release and caspase-3 activity [2]. In addition, autophagy may facilitate macromolecule degradation to compensate for the depletion of energy in brain tissue after stroke. However, the exact protective mechanism of autophagy in RPostC needs to be further elucidated.

Figure 6 Suppressing AKT/GSK3 β by LY294002 activates caspase-3 in limb RPostC rats. The expression of activated caspase-3 was assessed after 22-h reperfusion in sham, I/R, I/R + R-0, and I/R + R-0+LY294002 animals. **(A)** immunostaining for cleaved caspase-3 (brown) in paraffin-embedded slices from each group. Arrowheads indicate the cleaved caspase-3-positive cells. **(B)** Western blotting for cleaved caspase-3. **(C)** Statistical data showing that suppressing AKT/GSK3 β by LY294002 significantly activates caspase-3 in limb RPostC rats. Data are expressed as mean $\pm$ SEM from four animals. * P < 0.05 versus sham group, # P < 0.05 versus the I/R group. Scale bar = 10 μ m.

In conclusion, our results indicate that limb RPostC applied in the early phase of reperfusion in rats after MCAO reduces infarct size and improves neurological functions, upregulates neuron autophagy, and downregulates activated caspase-3, both of which require the involvement of the AKT/GSK3 β signaling pathway. The results of the present study may contribute to optimizing the protocol of limb RPostC for clinical use and provide insight for better understanding the mechanism underlying limb RPostC.

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Conflict of Interest

The authors declare no conflicts of interest.

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