Delayed remote ischemic postconditioning (DRIPost) has been shown to protect the rat brain from ischemic injury. However, extremely short therapeutic time windows hinder its translational use and the mechanism of action remains elusive. Because opening of the mitochondria K<sub>ATP</sub> channel is crucial for cell apoptosis, we hypothesized that the neuroprotective effect of DRIPost may be associated with K<sub>ATP</sub> channels. In the present study, the neuroprotective effects of DRIPost were investigated using adult male Sprague-Dawley rats. Rats were exposed to 90 minutes of middle cerebral artery occlusion followed by 72 hours of reperfusion. Delayed remote ischemic post-conditioning was performed with three cycles of bilateral femoral artery occlusion/reperfusion for 5 minutes at 3 or 6 hours after reperfusion. Neurologic deficit scores and infarct volumes were assessed, and cellular apoptosis was monitored by terminal deoxynucleotidyl transferase nick-end labeling. Our results showed that DRIPost applied at 6 hours after reperfusion exerted neuroprotective effects. The K<sub>ATP</sub> opener, diazoxide, protected rat brains from ischemic injury, while the K<sub>ATP</sub> blocker, 5-hydroxydecanote, reversed the neuroprotective effects of DRIPost. These findings indicate that DRIPost reduces focal cerebral ischemic injury and that the neuroprotective effects of DRIPost may be achieved through opening of K<sub>ATP</sub> channels.

Keywords: brain ischemia; K<sub>ATP</sub>; remote ischemic postconditioning; reperfusion injury

Introduction

Stroke is the third leading cause of death in the United States. Approximately 795,000 people experience a stroke and > 143,579 people die each year. Of all strokes, 87% are ischemic (Lloyd-Jones et al., 2010). The current lack of clinical treatment for acute stroke necessitates the exploration of novel concepts that may eventually lead to clinical application.
Materials and methods

Animals

Male Sprague-Dawley rats weighing 290 to 310 g were provided by the Experimental Animal Centre of the Fourth Military Medical University and housed under diurnal lighting conditions (12 hours darkness/light). All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Diazoxide (DIAY) and 5-hydroxydecanoate (5-HD) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Focal Cerebral Ischemia

Sprague-Dawley rats were allowed free access to food and water but were fasted 12 hours before surgery. All animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg in normal saline). The transient middle cerebral artery (MCA) occlusion model was performed as previously described (Xiong et al, 2007). Briefly, the right common carotid artery (CCA) and the right external carotid artery were exposed through a ventral midline neck incision, and were ligated proximally. A 3-0 nylon monofilament suture (Ethicon Inc., Osaka, Japan) with a blunt tip made by burning on a flame was inserted through the arterectomy in the CCA just below the carotid bifurcation, positioned into the internal carotid artery and advanced ~17 to 18 mm until a mild resistance was felt. Under these conditions, the origin of the MCAs was occluded. Reperfusion was accomplished by withdrawing the suture after 90 minutes of ischemia. The incision sites were infiltrated with 0.25% (v/v) bupivacaine hydrochloride for postoperative analgesia.

Regional cerebral blood flow was monitored using a flexible optical fiber probe attached to the skull over the ipsilateral parietal cortex at one point (1 mm posterior and 5 mm lateral to bregma) by laser Doppler flowmetry (PeriFlux system 5000; Perimed AB, Stockholm, Sweden). Rats in which ipsilateral blood flow was not reduced to <20% of the baseline after placement of the intraluminal filament and whose cerebral blood flow signal was not rapidly restored during reperfusion were excluded from subsequent experiments. Cranial temperature was maintained at 36.8°C to 37.5°C by surface heating and cooling during surgery. In a separate experiment, physiologic parameters (cranial temperature, arterial pH, PaCO2, PaO2, and glucose) were monitored and analyzed in five additional rats. Arterial blood samples were taken 3 minutes before ischemia (baseline), 45 minutes after ischemia, and 30 minutes after reperfusion and DRIPost for gases and plasma glucose measurements.

Infarct Volume Measurement

For measurements of infarct volume at 72 hours after cerebral artery reperfusion, rats were killed and the brains remained unstained (white), whereas normal tissue was stained red. Photographs were taken using a digital camera (SONY T9; Sony Corporation, Tokyo, Japan) The infarct area was demarcated and analyzed by Photoshop software (Adobe Photoshop 8.0, Adobe Systems Incorporated, San Jose, CA, USA). To compensate for the effect of brain edema, the corrected infarct volume was calculated as follows: percentage of corrected infarct volume = ((contralateral hemisphere - corrected infarct volume) / contralateral hemisphere) * 100.
area/ ipsilateral hemisphere/ measured infarct area)/
contralateral hemisphere area) × 100%. After brain extraction,
animals observed to have experienced a subarachnoid hemorrhage were excluded from the study.

Experimental Protocol

Experiments were composed of two parts. The first part was designed to prove the neuroprotective effect of DRIPost and explore the optimal method of DRIPost. The second part was designed to investigate the role of K<sub>ATP</sub> channels in the neuroprotective effect of DRIPost in vivo.

Part 1: Neuroprotective Effect of Delayed Remote Ischemic Postconditioning Against Cerebral Ischemia–Reperfusion Injury

To explore the optimal parameters required to observe the protective effects of DRIPost, our pilot study tested 56 rats with 7 different serials of DRIPost performed at 3 or 6 hours after reperfusion (n = 8 for each). The experimental protocols are shown in Figure 1A. Control (Con) group rats were subjected to 90 minutes of ischemia followed by 72 hours of reperfusion. For the DRIPost group, the bilateral femoral arteries were separated below the bilateral groin ligament for later induction of femoral artery occlusion after 90 minutes of ischemia. The occlusion duration varied from 15 seconds, 5 minutes to 8 minutes, and bilateral femoral arteries were released for the same duration and repeated for three cycles.

Part 2: Effect of K<sub>ATP</sub> Channel Blocking/Opening on Delayed Remote Ischemic Postconditioning-Induced Neuroprotection

In part 2, we further studied the role of K<sub>ATP</sub> channels in the neuroprotective effect of DRIPost. Sixty-four adult male Sprague-Dawley rats were randomly divided into eight
groups. The experimental protocols are shown in Figure 1B. Delayed remote ischemic postconditioning (5 minutes ischemia/5 minutes reperfusion in bilateral femoral arteries, three cycles) was induced at 6 hours after reperfusion in the postconditioning groups. This protocol showed a superior outcome in part 1 of the study. To block K<sub>ATP</sub> channels, 20 mg/kg of 5-HD, which was dissolved in normal saline to a concentration of 12 mg/mL, was injected through the caudal vein 30 minutes before DRIPost. To open K<sub>ATP</sub> channels, 5 mg/kg of DIAZ, which was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 3 mg/mL, was administered through the caudal vein 20 minutes before DRIPost. To exclude the effect of administrated drugs on cerebral ischemia–reperfusion injury, two separate control groups were used, which were administered the same dosage of drugs without postconditioning. Vehicle groups (saline group and DMSO group) were also included to preclude any influence.

Terminal Deoxynucleotidyl Transferase Nick-End Labeling and Quantification of Apoptosis
Apoptosis was quantified using a commercially available fluorescent terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit, in accordance with the manufacturer protocol (Roche Diagnostics Corporation, Indianapolis, IN, USA). The sections were mounted with 50% (v/v) glycerol for examination under a fluorescence microscope. The total number of TUNEL-positive neurons in the right hemisphere was counted in three different fields for each section in a blind manner by light microscopy at ×400 magnification (BX51; Olympus, Tokyo, Japan), and data from five animals at each stage were averaged.

Statistical Analysis
All data, excepting NDSs, are expressed as the mean ± s.d. Physiologic parameters were analyzed by repeated-measures analysis of variance. The NDSs were expressed as median (range). The NDSs among different groups were compared by Kruskal–Wallis test. When Kruskal–Wallis test showed significant difference, the Mann–Whitney U-tests with Bonferroni correction were applied. The infarct volumes and TUNEL-positive neurons were compared among groups by one factor analysis of variance. P<0.05 was considered statistically significant.

Results
Physiologic Parameters and Regional Cerebral Blood Flow
No statistical significance was noted among different time points for any of the physiologic parameters including cranial temperature, blood gas, and glucose concentrations (Table 1). Physiologic parameters remained in the normal range during the experimental period. Monitoring of regional cerebral blood flow ensured successful MCA occlusion (Figures 1C and 1D).

Neurologic Deficit Scores
All rats survived until 72 hours after reperfusion. At 24, 48, and 72 hours after reperfusion, the NDS in the 3 h-5 min, 3 h-8 min, and 6 h-5 min groups was significantly higher than that in the Con group (P<0.05). Furthermore, the scores of animals in all groups were significantly lower than that of the 6 h-5 min group at each time point, which highlighted the neuroprotective effect of the 6 h-5 min DRIPost protocol in experiment part 1 (Figures 2A–2C).

In part 2 of the experiment, at 24, 48, and 72 hours after reperfusion, the NDS in the DIAZ, DRIPost, DRIPost + DIAZ, and DRIPost + 5-HD groups was significantly higher than that of the Con group (P<0.05). There was no statistical difference in NDS among the DRIPost and DRIPost + DIAZ groups at 24 and 48 hours after reperfusion (P=0.328 and 0.382, respectively). In contrast, the NDS in the DRIPost + 5-HD group was significantly lower than that of the DRIPost group at the three time points (P<0.05; Figures 3A–3C).

Infarct Volume
In experiment part 1, the infarct volume results are shown in Figure 2. All DRIPost protocols, except 3 h-15 s (60.48 ± 3.08%), significantly decreased infarct volumes at 72 hours after reperfusion compared with that of the Con group (63.88 ± 3.66%, P<0.05). Among the protocols implemented in this study, the neuroprotective effect of three cycles of 5 minutes occlusion/reperfusion at 6 hours after reperfusion was superior (34.64 ± 4.00%, P<0.05; Figures 2D and 2E).

In experiment part 2, the infarct volume results are shown in Figure 3. Infarct volume in the DRIPost
group (33.45 ± 2.78%) was significantly smaller than that of the Con group (65.58 ± 3.30%) at 72 hours after reperfusion (P = 0.000). Administration of DIAZ (DIAZ group and DRIPost + DIAZ group) resulted in a reduction in infarct volume (39.83 ± 5.04% and 34.36 ± 2.56%, respectively) compared with the Con group (P = 0.000). Administration of 5-HD (DRIPost + 5-HD) eliminated the neuroprotective effect of DRIPost (46.44 ± 2.65% versus 33.45 ± 2.78%, P = 0.000). However, there was no significant difference in infarct volume between the DRIPost and DRIPost + DIAZ groups (33.45 ± 2.78% versus 34.36 ± 2.56%, P = 0.602; Figures 3D and 3E).

**Figure 2** Neurologic scores and infarct volume after 90 minutes of transient middle cerebral artery occlusion in control (CON) and delayed remote limb ischemic postconditioning (DRIPost) groups in part 1 (n = 8 for each). DRIPost was performed with bilateral femoral artery occlusion/reperfusion at 3 or 6 hours after reperfusion. Neurologic scores are presented as the median (range); Data for infarct volumes are expressed as the mean ± s.d. Neurologic scores were evaluated at 24 hours (A), 48 hours (B), and 72 hours (C) after reperfusion using the Garcia scoring system. (D) Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in the rat brain at 72 hours after reperfusion. (E) Statistical analysis of the percentage of infarct volume was determined for each study group. 3 h/6 h-15 s, 5 min and 8 min groups: DRIPost performed at 3 or 6 hours after reperfusion. The occlusion duration varied from 15 seconds, 5 minutes to 8 minutes, and bilateral femoral arteries were released for the same duration and repeated for three cycles. *P < 0.05 versus Con group. *P < 0.05 versus 6 h-5 min group.

**Discussion**

This study showed that RIPost with three cycles of 5 minutes limb ischemia/5 minutes reperfusion, which was conducted even 6 hours after focal cerebral ischemia–reperfusion injury, could reduce infarct size and improve neurologic deficits. Ischemia–reperfusion-induced neuronal apoptosis was also markedly attenuated by DRIPost. Activation of K<sub>ATP</sub> channels is critical for endothelial protection by DRIPost because the conditioning stimuli were ineffective when applied in the presence of the nonselective K<sub>ATP</sub> channel blocker 5-HD. Taken together, the

**Terminal Deoxynucleotidyl Transferase Nick-End Labeling**

The number of TUNEL-positive neurons in the CA<sub>1</sub> region significantly increased at 72 hours after ischemia–reperfusion injury in the Con group (Figure 4Aa). At the same observation time, the number of TUNEL-positive neurons in the CA<sub>1</sub> region of the DRIPost group was significantly lower than that in the Con group (P < 0.05; Figure 4Ad). Injection of 5-HD, a K<sub>ATP</sub> blocker, before DRIPost, increased the number of TUNEL-positive neurons in the CA<sub>1</sub> region after reperfusion (Figure 4Ae). However, there was no significant difference in the number of apoptotic neurons between the DRIPost and DRIPost + DIAZ groups 72 hours after reperfusion (Figure 4Ad and f).
present study provides evidence that the neuroprotective effect of DRIPost is associated with the activation of K<sub>ATP</sub> channel opening.

We found that the optimal procedure for DRIPost, performed a few hours later, differed from that of rapid RIPost. Currently, there are at least two types of IPost, conventional IPost and RIPost. The conventional IPost refers to a series of brief, repetitive mechanical occlusions/reperfusions at the onset of reperfusion after long-term ischemia (Zhao et al., 2003). As for brain injury, conventional IPost was first reported by Zhao et al. (2006) in a rat cerebral ischemia model. This protective concept of IPost has been confirmed by a number of groups using in-vivo global and focal ischemia models (Gao et al., 2008; Pignataro et al., 2008b) and in-vitro ischemic models (Pignataro et al., 2008b). Despite evidence from numerous animal studies, clinical application of in-situ ischemia for postconditioning of the brain is quite an unacceptable concept and impracticable. Recently, RIPost was primarily verified in studies of myocardial injury. Both limb ischemic (Gritsopoulos et al., 2009; Tang et al., 2010) and renal IPost showed potential cardiac protective effects (Liu et al., 2007). A recent study reported that RIPost reduced cerebral infarction by 67% (Ren et al., 2009). In addition, DRIPost initiated as late as 3 hours, but not 6 hours, still robustly reduced brain infarction by 43%. In our study, we found that three cycles of postischemic treatment, either 5 minutes occlusion/5 minutes reperfusion or 8 minutes occlusion/8 minutes reperfusion for each circle, were effective for inducing neuroprotection either applied at 3 hours or 6 hours after reperfusion. Among the effective procedures, 6 h-5 min group showed greater potential in reducing tissue damage after reperfusion. The different results between our group and Zhao's report may mainly reside in the difference of animal model. Taken together, both results suggest that early reperfusion after brain ischemic injury may broadly widen the therapeutic window for DRIPost, thus providing a better chance of neural salvage.
The underlying protective mechanisms of DRIPost are unknown. Nevertheless, our previous research from remote ischemic preconditioning (RIPC) against cerebral and spinal cord ischemia may shed light on our understanding of the protective mechanisms of DRIPost (Dong et al., 2010; Su et al., 2009). Emerging evidence from animal studies suggests that RIPC, IPost, and ischemic preconditioning share common signaling pathways, including triggers (adenosine receptor stimulation) (Kerendi et al., 2005; Steensrud et al., 2010), mediators (protein kinase C activation), and end effectors (opening of mitochondrial K<sub>ATP</sub> channels, activation of prosurvival kinases, and inhibition of mitochondrial permeability transition pore opening; Hausenloy and Yellon, 2009; Meier et al., 2005; Yang et al., 2004). A popular theory has proposed that a substance or humoral factor, such as oxygen free radicals, is carried in the blood stream from the preconditioning organ or tissue to the organ where it manifests its protective effect. After humoral factors arrive to the remote organs, the end effectors (such as K<sub>ATP</sub> channels) appear to be activated and have an important role in the induction of ischemic tolerance by RIPC. The protective effect of K<sub>ATP</sub> has been showed in several tissues, including the intestines, kidneys, liver, and brain (Hai et al., 2005; Tawfik et al., 2009). Opening of K<sub>ATP</sub> channels has been shown to be a prerequisite event for the induction of protection against ischemia–reperfusion injury by RIPC, IPost, and RIPost (Schmidt et al., 2007; Steensrud et al., 2010; Yang et al., 2004). Zhang et al (2010) used an in-vitro model of PC12 cells

![Figure 4](image)

Figure 4 (A) Representative sections of nuclear DNA fragmentation staining performed by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) in the CA1 region of the hippocampus from rats that randomly received 5-HD (20 mg/kg, intravenously) or DIAZ (5 mg/kg, intravenously) after 90 minutes of ischemia and 72 hours of reperfusion (Con). In the presence and absence of 5-HD or DIAZ pretreatment, or with a combination of DRIPost (three cycles of 5 minutes occlusion/reperfusion at 6 hours after reperfusion) and 5-HD or DIAZ. (A) Control (Con); (B) 5-HD; (C) DIAZ; (D) DRIPost; (E) DRIPost + 5-HD; (F) DRIPost + DIAZ. (B) Quantitative analysis of the number of TUNEL-positive neurons in the CA1 region of the hippocampus. Data are presented as the mean ± s.d. Scale bar, 20 μm. *P < 0.05 versus Con group. *P < 0.05, versus DRIPost group. DIAZ, diazoxide; 5-HD, 5-hydroxydecanate.
undergoing oxygen glucose deprivation to reveal that activation of $K_{\text{ATP}}$ channels elicits protective effects against oxygen glucose deprivation-induced cell apoptosis by caspase-dependent and -independent mitochondrial pathways. In addition, Loukogeorgakis et al. (2007) revealed that transient limb ischemia induces RIPost in humans by a $K_{\text{ATP}}$ channel-dependent mechanism. Thus, we speculate that $K_{\text{ATP}}$ may also be involved in the neuroprotective effect of DRIPost. In our study, DIAZ, an activator of $K_{\text{ATP}}$ channels, significantly reduced morphological injury, TUNEL-positive cells, and neurologic deficiency after cerebral ischemia–reperfusion injury. However, the $K_{\text{ATP}}$ blocker 5-HD reversed the neuroprotective effect of DRIPost. These results indicate that the activation of $K_{\text{ATP}}$ is involved in the neuroprotective effect of DRIPost.

Several limitations of this study merit comments. First, only six different protocols were designed in this study. The results showed that DRIPost is still effective when applied at 6 hours after reperfusion. Whether the same results can be drawn at even later phases after injury or protocols where other parameters are altered (i.e., changes in the frequency of I/R cycle, application time or duration of occlusion and reperfusion of limb postconditioning) show more potent neuroprotection deserves further elucidation. Our results only exhibited a partial reversal of neuroprotection induced by DRIPost after 5-HD injection, suggesting that opening of $K_{\text{ATP}}$ channels is not the only cause for the protective effect. In addition, differences could be due to different animal models (heart or brain ischemia–reperfusion injuries) used. Both timing and length of IPost have been confirmed to be important in determining the magnitude of its protective effect. Ren et al. (2008) also found that the protective effect of limb RIPC depends on the number and duration of the limb ischemic stimulus. Previous studies have shown that endogenous activation of adenosine receptors, especially the A$_2$A and A$_3$A subtypes, is involved in IPost- and RIPC-mediated cardiac protection (Kerendi et al., 2005; Tsubota et al., 2010). Wang et al. (2011) revealed that RIPost performed in one limb alleviated reperfusion injury after focal cerebral ischemia through reactive oxygen species-mediated inhibition of endogenous $\delta$ protein kinase C activation signaling cascade in an in-vivo rat model of focal cerebral ischemia. Whether other mediators and effectors such as protein kinase C-E and adenosine A$_1$ receptors that mediate the protective effect of IPost in myocytes could also participate in the induction of ischemic tolerance by DRIPost in the brain remains to be clarified.

In conclusion, DRIPost, even when applied at 6 hours after reperfusion, can induce a potent neuroprotective effect against focal cerebral ischemic reperfusion injury and inhibit apoptotic injury in the ischemic brain. The protective effects may partially be due to opening of $K_{\text{ATP}}$ channels. Delayed remote ischemic postconditioning may be established as a potential practicable treatment for comparatively late-hour ischemic stroke patients who receive medical care hours after the onset of stroke.

**Disclosure/Conflict of interest**

The authors declare no conflict of interest.

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