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Neuroprotection against Spinal Cord Ischemia–Reperfusion Injury Induced by Different Ischemic Postconditioning Methods

Roles of Phosphatidylinositol 3-Kinase–Akt and Extracellular Signal–regulated Kinase

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Background: **The authors compared the neuroprotective effects induced by two ischemic postconditioning methods and sought to determine the roles of phosphatidylinositol 3-kinase–Akt and extracellular signal–regulated kinase (ERK) in this neuroprotection.**

Methods: **Spinal cord ischemia was induced in rabbits by occlusion of the infrarenal aorta with a balloon catheter for 25 min. Postconditioning was accomplished by either five cycles of 1-min occlusion and 1-min reperfusion (standard postconditioning) or control of the perfusion pressure between 45 and 55 mmHg at the first 10 min of reperfusion (modified postconditioning). Motor function was assessed with the Tarlov score during a 28-day observation period. Histologic examination of lumbar spinal cords was performed. Expressions of Akt and ERK in the spinal cord were evaluated by Western blot.**

Results: **Compared with the controls, the two postconditioning methods markedly increased Tarlov scores 1, 3, 7, and 28 days after spinal cord ischemia and number of intact motor neurons in the lumbar spinal cord. No significant difference in Tarlov scores and number of intact motor neurons was detected between the two postconditioning method groups. The two postconditioning methods enhanced the expressions of phospho-Akt and phospho-ERK in spinal cords. The neuroprotective effects and the increases in phospho-Akt and phospho-ERK were abolished by administration of phosphatidylinositol 3-kinase–Akt inhibitor LY-294002 or ERK inhibitor PD-98059.**

Conclusions: **The two postconditioning methods possess comparable neuroprotective effects on the spinal cord and share a common molecular mechanism, in which phosphatidylinositol 3-kinase and ERK pathways play crucial roles.**

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PARAPLEGIA remains a major devastating and unpredictable complication after surgical repair of descending and thoracoabdominal aneurysms, with the reported incidence ranging from 6.6% to 8.3%.[1,2] Although multiple factors contribute to this complication, the principal root is thought to be the ischemia and reperfusion injury of the spinal cord.[1,3]

Ischemic preconditioning, which was first described as a paradoxical form of cardioprotection,[4] has now been universally accepted to be a potent endogenous protection to enhance the tolerance against ischemia in several organs, including the spinal cord.[5,6] A novel approach to myocardial protection, named as *ischemic postconditioning*, was recently reported by Vinten-Johansen's group, in which brief intermittent repetitive interruptions to reperfusion at the onset of reperfusion after a prolonged period of ischemia reduced myocardial injury to an extent comparable to ischemic preconditioning.[7] The cardioprotective effects of ischemic postconditioning have been further confirmed in different myocardial ischemic models and species.[8–10] In the latest researches, ischemic postconditioning has been shown to be a novel neuroprotective strategy against cerebral ischemia–reperfusion injury.[11–14] However, little is known about whether this endogenous neuroprotection is also effective in spinal cords. In our previous study, postconditioning consisting of four to six cycles of 1-min occlusion and 1-min reperfusion applied at the start of reperfusion was first reported to attenuate neurologic injury resulting from spinal cord ischemia, and the first a few minutes of reperfusion were crucial to its neuroprotection.[15] In a further study, the perfusion pressure was controlled between 45 and 55 mmHg during the first 10 min followed by complete reperfusion, and the neurologic injuries after spinal cord ischemia were also reduced.[16] Because the protective intervention targeted the onset of reperfusion like the postconditioning, this method may be regarded as modified postconditioning. Consistent with our results, gradual reperfusion generated by controlled release bilateral common carotid

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arteries was demonstrated to induce robust neuroprotective effects in a rat model of cerebral ischemia.[12]

Although the neuroprotection of ischemic postconditioning has been confirmed in collective studies, the underlying molecular mechanisms are still not fully understood. Activation of the prosurvival kinase phosphatidylinositol 3-kinase (PI3K)–Akt and the mitogen-activated protein kinase p44/p42 extracellular signal–regulated kinase (ERK) at the time of reperfusion together comprise the cardioprotective reperfusion injury salvage kinase pathway, which has been shown to play an important role in the induction of myocardial protection afforded by ischemic posctconditioning.[10,17] PI3K–Akt and/or ERK pathways were also found to contribute to the neuroprotection against brain ischemia generated by pharmacologic intervention, ischemic preconditioning, and ischemic postconditioning.[13,18–20] It is still not known whether PI3K–Akt and/or ERK pathways are involved in the spinal cord protection afforded by ischemic postconditioning.

In the current study, postconditioning was performed by either repetitive brief occlusion and reperfusion after the start of reperfusion (standard postconditioning) or controlled low-pressure perfusion at the beginning of reperfusion (modified postconditioning) in a rabbit model of spinal cord ischemia. We compared the neuroprotective effects induced by these two methods of ischemic postconditioning and tried to test the hypothesis that ischemic postconditioning mediates its neuroprotection on the spinal cord by activation of classic survival kinases, such as PI3K and ERK.

Materials and Methods

Animals

Male Japanese white rabbits weighing 1.8–2.5 kg were used in the study. The animal protocol was approved by the Ethics Review Committee for Animal Experimentation of China Medical University (Shenyang, P. R. China) and was in accordance with the *Guide for the Use and Care of Laboratory Animals* (National Institutes of Health, Bethesda, Maryland).

Surgical Procedure

Surgery was conducted according to the method described previously.[16] Briefly, the rabbits were anesthetized with intravenous sodium pentobarbital (25 mg/kg). Core body temperature was continuously monitored with a rectal probe and was maintained at 38.5° ± 0.5°C with the aid of a heating lamp. A 22-gauge catheter was inserted into the ear artery to measure proximal blood pressure. A 4-French balloon-tipped catheter (Goodtec Inc., Huntington Beach, CA) was inserted through an arteriotomy in the left femoral artery and advanced 15 cm retrograde into the abdominal aorta. Preliminary investigations confirmed that the balloon should be positioned 0.5 to 1.2 cm distal to the left renal artery.[21] After systemic heparinization (200 U/kg), spinal cord ischemia was induced by inflation of the balloon. Complete aortic occlusion was confirmed by reduction in distal aortic blood pressure to less than 20 mmHg, which was measured through the side hole of the balloon catheter. At the end of the operation, the catheter was removed and the femoral artery was reconstructed. Arterial blood gas analysis and blood glucose were recorded before ischemia (baseline), 15 min after ischemia, and 20 min after reperfusion.

Intrathecal Injection

Before the balloon catheter was placed, the intervertebral space between L5 and L6 was punctured with a 16-gauge needle, and polyethylene-10 tubing was inserted through it into the subarachnoid space. The desired position of the catheter was confirmed by cautious aspiration of cerebrospinal fluid. After intrathecal injection of vehicle or the drugs, the catheter was removed.[22]

Experimental Protocol

All rabbits except the sham animals were subjected to 25 min of spinal cord ischemia. Six rabbits were assigned randomly to each of the following 10 groups, as shown in figure 1:

1. Sham group: Rabbits underwent the same surgical procedure and intrathecal injection of 10% dimethyl sulfoxide (DMSO), 20 μl, without aortic occlusion.
2. Control, LY (LY-294002), or PD (PD-98059) groups: Intrathecal injection of 10% DMSO, 20 μl; PI3K–Akt inhibitor LY-294002, 20 μl (0.1% in 10% DMSO); or ERK inhibitor PD-98059, 20 μl (0.015% in 10% DMSO), was conducted 1 min before the full reperfusion.
3. Postcon A (standard postconditioning), Postcon A + LY, Postcon A + PD: After 25 min of ischemia, full reperfusion was initiated for 1 min followed by 5 cycles of postconditioning (one cycle comprised 1-min occlusion and 1-min reperfusion). Intrathecal injection of DMSO, 20 μl; LY-294002, 20 μl (0.1% in 10% DMSO); or PD-98059, 20 μl (0.015% in 10% DMSO), was conducted 1 min before the start of reperfusion.
4. Postcon B (modified postconditioning), Postcon B + LY, Postcon B + PD: During the first 10 min of reperfusion, blood flow was partially restored and the mean blood pressure of the distal aorta was controlled between 45 and 55 mmHg by adjusting the volume of the balloon. Then, complete reperfusion was performed. Intrathecal injection of DMSO, 20 μl; LY-294002, 20 μl (0.1% in 10% DMSO); or PD-98059, 20 μl (0.015% in 10% DMSO), was conducted 1 min before the partial reperfusion.

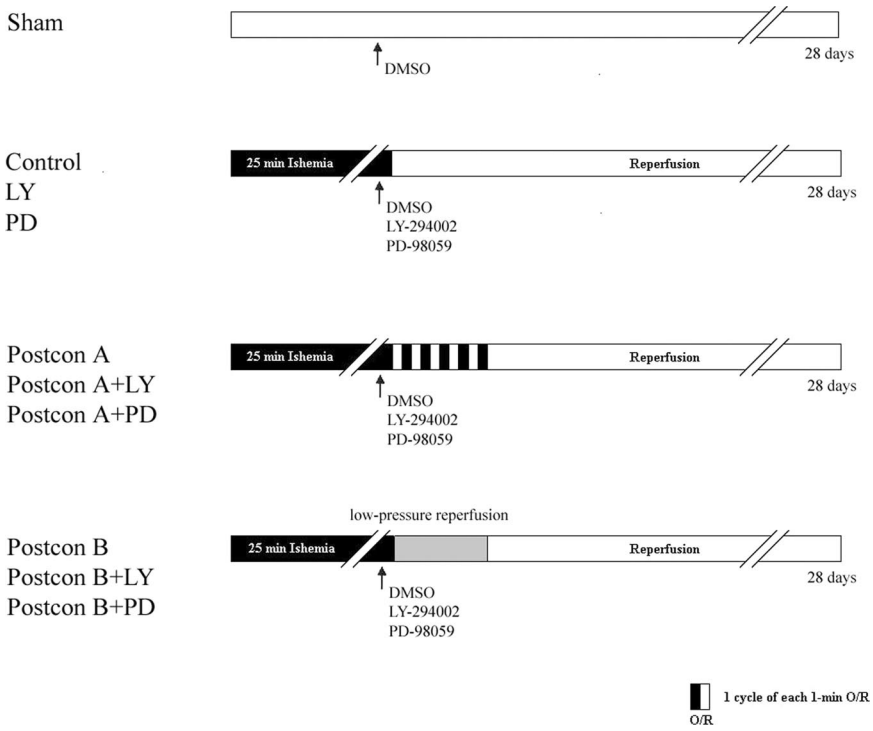

Fig. 1. Experimental groups and protocol. DMSO = dimethyl sulfoxide; LY = LY-294002; O = occlusion; PD = PD-98059; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion); R = reperfusion.

Neurologic Assessment

During a 28-day recovery after ischemia, hind-limb motor function was assessed by two blinded observers using a modified Tarlov scale[23]: 0, paraplegic with no evident lower extremity motor function; 1, poor lower extremity motor function, weak antigravity movement only; 2, moderate lower extremity function with good antigravity strength but inability to draw legs under body; 3, excellent motor function with the ability to draw legs under body and hop, but not normally; and 4, normal motor function.

Histologic Study

For histologic study, rabbits were killed 28 days after the transient spinal cord ischemia. Paraffin-embedded sections (4 μm) of lumbar spinal cords (L4–L6) were stained with hematoxylin–eosin. In cases in which the cytoplasm was diffusely eosinophilic, the large motor neuron cells were considered to be necrotic or dead. When the cells demonstrated basophilic stippling (containing Nissl substance), the motor neuron cells were considered to be viable or alive.[24] The intact motor neurons in the ventral gray matter were counted by a blinded investigator in three sections for each rabbit, and the results were then averaged.

Western Blot

In a parallel series of experiments, spinal cords were collected 2 h after reperfusion from the following groups: control, Postcon A, Postcon B, LY, Postcon A + LY, Postcon B + LY, PD, Postcon A + PD, and Postcon B + PD (n = 4/group). Total Akt, phosphorylation states of Akt (phospho-Akt), total ERK, and phosphorylation states of ERK (phospho-ERK) proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis using specific antibodies as described previously.[25] The optical density for each band on the Western blot was quantified using the National Institutes of Health image program and was normalized to the corresponding Ponceau stain signal.

Statistical Analysis

Parametric values are reported as mean ± SD. The Kruskal–Wallis test was used for nonparametric values (Tarlov score and number of neurons), and the Mann–Whitney U test was used as a posttest to identify the specific differences between the groups. Parametric values were analyzed by one-way or two-way repeated-measures (time and group) analysis of variance followed by the Student–Newman–Keuls *post hoc* test. Statistical significance was defined as $P < 0.05$.

Results

A total of 64 rabbits were enrolled in the current study. Two rabbits were excluded as a result of technical difficulties with the preparation of spinal cord ischemia model. Another 2 rabbits were omitted because of death 1 and 4 days after the surgery.

Physiologic Parameters

Table 1 indicates the physiologic parameters from all the groups. The mean blood pressure of distal aorta of all

Table 1. Physiologic Parameters

	Sham	Control	Postcon A	Postcon B	LY	Postcon A + LY	Postcon B + LY	PD	Postcon A + PD	Postcon B + PD
Body weight, kg	2.1 ± 0.2	2.2 ± 0.4	2.0 ± 0.2	2.1 ± 0.3	2.3 ± 0.4	2.2 ± 0.2	2.2 ± 0.3	2.0 ± 0.4	2.1 ± 0.2	2.2 ± 0.2
Proximal MAP, mmHg										
Baseline	92.3 ± 8.5	91.7 ± 7.8	91.8 ± 6.9	90.4 ± 11.2	92.1 ± 7.7	90.2 ± 6.5	89.7 ± 5.1	94.5 ± 8.9	92.3 ± 4.3	92.3 ± 5.1
Ischemia 15 min		94.1 ± 5.6	92.3 ± 5.8	92.3 ± 8.7	91.3 ± 6.3	89.9 ± 4.5	90.2 ± 5.8	91.2 ± 3.3	91.6 ± 7.4	93.4 ± 6.8
Reperfusion 20 min		85.7 ± 7.2	84.4 ± 6.4	85.1 ± 3.7	86.4 ± 5.9	85.4 ± 5.4	88.4 ± 6.1	91.3 ± 4.7	87.1 ± 8.9	87.4 ± 7.4
Distal MAP, mmHg										
Baseline	91.7 ± 11.3	92.3 ± 8.7	90.5 ± 7.0	89.8 ± 8.6	91.5 ± 8.1	90.7 ± 7.2	88.9 ± 9.1	93.1 ± 11.4	90.7 ± 5.1	89.7 ± 6.6
Ischemia 15 min		15.7 ± 2.7*	15.8 ± 1.7*	14.7 ± 2.2*	16.2 ± 5.5*	14.8 ± 3.8*	17.6 ± 1.9*	15.7 ± 2.1*	14.5 ± 1.0*	13.5 ± 0.9*
Reperfusion 20 min		85.3 ± 13.8	87.5 ± 5.6	85.9 ± 3.3	84.6 ± 7.7	87.9 ± 8.1	89.7 ± 5.6	90.7 ± 11.5	86.4 ± 9.2	84.1 ± 6.5
HR, beats/min										
Baseline	262.0 ± 20.4	248.3 ± 29.9	262.7 ± 20.8	255.3 ± 17.0	249.5 ± 21.5	257.8 ± 30.7	253.6 ± 21.5	265.7 ± 19.2	243.8 ± 23.3	259.4 ± 18.8
Ischemia 15 min		261.0 ± 20.6	263.3 ± 20.4	260.8 ± 13.0	255.7 ± 16.1	247.8 ± 23.4	260.4 ± 25.1	261.5 ± 19.7	249.5 ± 18.8	263.3 ± 23.4
Reperfusion 20 min		256.2 ± 19.1	269.8 ± 17.1	261.4 ± 14.7	257.8 ± 19.9	260.4 ± 27.4	257.9 ± 30.5	249.4 ± 18.9	255.5 ± 25.0	258.9 ± 26.1
Rectal temperature, °C										
Baseline	38.7 ± 0.5	38.5 ± 0.3	38.4 ± 0.3	38.7 ± 0.4	38.9 ± 0.2	38.6 ± 0.4	38.7 ± 0.1	38.6 ± 0.1	38.7 ± 0.2	38.8 ± 0.3
Ischemia 15 min		38.7 ± 0.3	38.6 ± 0.1	38.9 ± 0.3	38.7 ± 0.4	38.3 ± 0.5	38.4 ± 0.4	38.6 ± 0.2	38.6 ± 0.3	38.2 ± 0.5
Reperfusion 20 min		38.5 ± 0.5	38.6 ± 0.4	38.6 ± 0.2	38.7 ± 0.1	38.9 ± 0.3	38.8 ± 0.4	38.7 ± 0.4	38.1 ± 0.3	38.2 ± 0.4
pH										
Baseline	7.36 ± 0.03	7.38 ± 0.04	7.37 ± 0.03	7.38 ± 0.05	7.33 ± 0.04	7.31 ± 0.02	7.39 ± 0.04	7.37 ± 0.03	7.35 ± 0.02	7.34 ± 0.05
Ischemia 15 min		7.39 ± 0.02	7.38 ± 0.05	7.39 ± 0.05	7.36 ± 0.03	7.40 ± 0.05	7.36 ± 0.01	7.38 ± 0.03	7.34 ± 0.02	7.39 ± 0.03
Reperfusion 20 min		7.40 ± 0.03	7.35 ± 0.04	7.38 ± 0.04	7.39 ± 0.02	7.33 ± 0.03	7.41 ± 0.04	7.36 ± 0.04	7.37 ± 0.04	7.38 ± 0.05
P_{O_2}, mmHg										
Baseline	99.8 ± 22.3	113.8 ± 33.8	104.8 ± 13.1	109.5 ± 15.1	110.2 ± 22.0	104.6 ± 13.2	109.8 ± 18.1	97.5 ± 17.4	104.2 ± 16.3	100.3 ± 14.1
Ischemia 15 min		111.8 ± 36.3	92.6 ± 20.6	99.8 ± 21.7	98.9 ± 14.6	107.3 ± 13.8	100.7 ± 19.4	110.2 ± 15.4	101.7 ± 19.6	106.8 ± 9.8
Reperfusion 20 min		111.4 ± 25.3	95.4 ± 16.8	99.0 ± 21.7	116.5 ± 20.7	107.8 ± 11.3	102.4 ± 17.8	109.6 ± 12.3	106.7 ± 18.9	112.2 ± 14.4
P_{CO_2}, mmHg										
Baseline	34.8 ± 2.9	33.8 ± 2.9	35.0 ± 4.9	37.2 ± 3.0	35.4 ± 7.2	33.8 ± 6.7	37.5 ± 7.1	35.2 ± 8.6	39.6 ± 7.8	35.1 ± 4.5
Ischemia 15 min		34.4 ± 5.9	36.2 ± 2.8	35.6 ± 1.6	39.4 ± 3.3	40.3 ± 4.3	36.6 ± 5.2	33.7 ± 6.9	39.7 ± 7.4	37.4 ± 6.7
Reperfusion 20 min		33.6 ± 3.6	36.0 ± 2.7	37.8 ± 4.8	32.5 ± 5.8	34.4 ± 7.1	33.7 ± 8.3	35.7 ± 5.8	37.1 ± 4.8	34.5 ± 6.7
Glucose, mmol										
Baseline	5.9 ± 1.1	6.1 ± 0.8	6.0 ± 0.9	5.8 ± 0.9	6.1 ± 1.6	6.0 ± 0.8	5.8 ± 0.7	6.2 ± 1.3	5.9 ± 1.1	6.3 ± 1.2
Ischemia 15 min		6.1 ± 1.4	6.2 ± 1.1	6.2 ± 1.4	6.2 ± 1.2	6.2 ± 1.3	6.0 ± 1.5	6.2 ± 1.8	6.0 ± 1.3	6.1 ± 0.8
Reperfusion 20 min		6.3 ± 1.2	6.3 ± 1.4	6.1 ± 1.2	6.3 ± 1.4	6.1 ± 0.8	6.1 ± 1.1	6.1 ± 0.9	6.2 ± 1.4	6.2 ± 1.4

Values are given as mean ± SD.

* $P < 0.05$ compared with baseline.

HR = heart rate; LY = LY-294002; MAP = mean arterial pressure; P_{CO_2} = partial pressure of carbon dioxide; PD = PD-98059; P_{O_2} = partial pressure of oxygen; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion).

the groups except the sham group was significantly decreased during the aortic occlusion ($P < 0.01$ *vs.* baseline). There were no significant differences in body weight, hemodynamics, rectal temperature, blood gas analysis data, or blood glucose among the groups at any time point ($P > 0.05$).

Neurologic Assessment

The individual neurologic scores of the 10 groups 1, 3, 7, and 28 days after the operation are shown in figure 2. The sham animals retained a normal motor function of lower limbs throughout the observation period. A 25-min aortic occlusion resulted in severe lower extremity neurologic deficits in the control rabbits, whereas the two postconditioning methods remarkably enhanced the motor function of the lower limbs after spinal cord ischemia as indicated by the significantly higher Tarlov scores of the Postcon A and Postcon B groups at the four observation time points ($P < 0.05$ *vs.* control group). Equivalent Tarlov scores were observed between the Postcon A group and Postcon B group at the four observation time points ($P > 0.05$). The neuroprotective effects induced by the two postconditioning methods were completely abolished by intrathecal injection of LY-294002 or PD-98059. The Tarlov scores of Postcon A + LY and Postcon A + PD were significantly lower than those of Postcon A ($P < 0.05$). The Tarlov scores of Postcon B + LY and Postcon B + PD were significantly lower than those of Postcon B ($P < 0.05$). No significant differences were detected between the Tarlov scores of the control group with those of the LY group or PD group at each time point ($P > 0.05$).

Histologic Assessment

Representative sections of lumbar spinal cords stained with hematoxylin–eosin are shown in figure 3A, and the results of counting viable motor neurons are summarized in figure 3B. In the sham-operated animals, the spinal cord was intact, and many large motor neurons were present in the anterior horn. A 25-min aortic occlusion induced severe neurologic damage in animals of the control group 28 days after ischemia, as indicated by vacuolization, frank necrosis, and an almost total loss of motor neurons. Similar histologic damages were found in the following groups: LY, PD, Postcon A + LY, Postcon A + PD, Postcon B + LY, and Postcon B + PD.

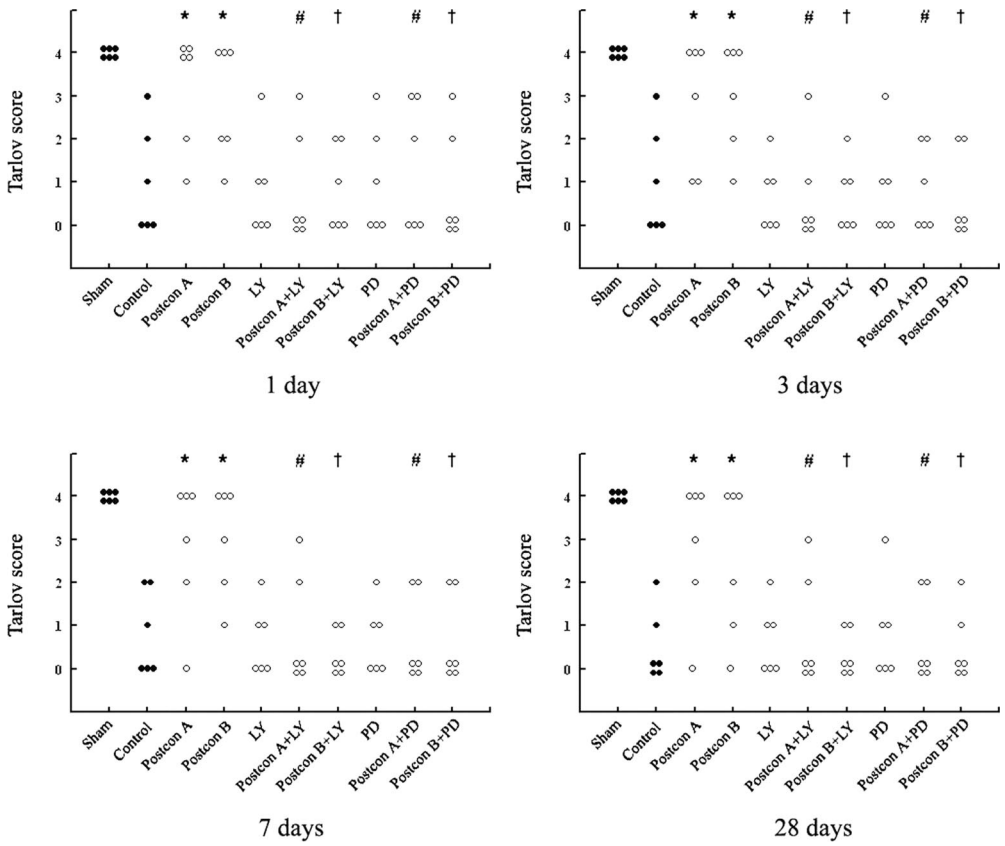

Fig. 2. Motor function assessed with Tarlov score at 1, 3, 7, and 28 days after transient spinal cord ischemia. *Circles* represent individual rabbit. LY = LY-294002; PD = PD-98059; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion). * $P < 0.05$ *versus* control group. # $P < 0.05$ *versus* Postcon A group. † $P < 0.05$ *versus* Postcon B group.

There were no significant differences in the intact motor neuron count of these groups compared with that of the control group ($P > 0.05$). In contrast, slighter histologic changes were detected in lumbar spinal cords of animals in the Postcon A and Postcon B groups, and the intact motor neurons were preserved to a much greater extent ($P < 0.05$ *vs.* control group). No significant difference in the number of motor neurons was detected between the Postcon A and Postcon B groups ($P > 0.05$).

Expression of Akt and ERK

Compared with the control animals, the two postconditioning methods resulted in a significantly higher expression of phospho-Akt, but not total Akt (in arbitrary units, Postcon A 61.7 ± 8.6, Postcon B 67.8 ± 11.6 *vs.* control 44.0 ± 9.6; $P < 0.05$). No significant difference in the expression of phospho-Akt was detected between the Postcon A and Postcon B groups (in arbitrary units, 61.7 ± 8.6 *vs.* 67.8 ± 11.6; $P > 0.05$). The increased expressions of phospho-Akt induced by the two postconditioning methods were completely abolished in the presence of PI3K inhibitor LY-294002 (in arbitrary units, Postcon A + LY 42.3 ± 16.0, Postcon B + LY 36.3 ± 12.1 *vs.* control 44.0 ± 9.6; $P > 0.05$). A representative Western blot image and the results of densitometric analysis are depicted in figure 4.

As illustrated in figure 5, the expressions of phospho-ERK, but not total ERK, were also markedly enhanced by the two postconditioning methods to an equivalent level, which was much higher than that of the control group (in arbitrary units, Postcon A 71.7 ± 6.9, Postcon B 74.8 ± 12.1 *vs.* control 53.0 ± 11.2; $P < 0.05$). Intrathecal injection of ERK inhibitor PD-98059 inhibited the phospho-ERK–increasing effects afforded by the two postconditioning methods (in arbitrary units, Postcon A + PD 49.8 ± 12.0, Postcon B + PD 39.3 ± 9.1 *vs.* control 53.0 ± 11.2; $P > 0.05$).

Discussion

The salient findings of the current study can be summarized as follows: (1) Partial restoration of the blood flow at the first 10 min of reperfusion by control of the perfusion pressure to 45–55 mmHg, which was regarded as modified ischemic postconditioning, protected the spinal cord against ischemia–reperfusion injury to an extent comparable to standard ischemic postconditioning. (2) The neuroprotective effects induced by the two postconditioning methods were abolished by pharmacologic inhibition of either PI3K–Akt or ERK. (3) The two postconditioning methods significantly enhanced the ex-

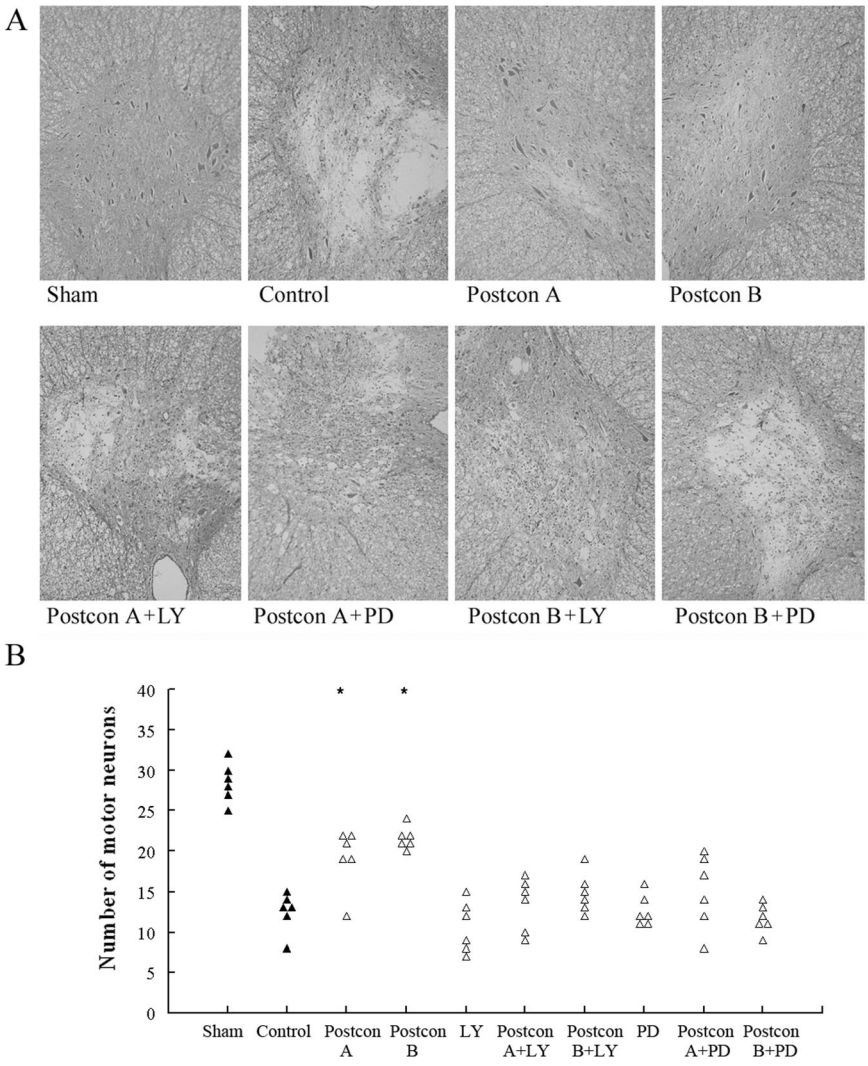

Fig. 3. Histologic assessment of the spinal cord 28 days after transient ischemia. (*A*) Representative sections of lumbar spinal cords stained with hematoxylin–eosin (magnification 100×). (*B*) Number of intact motor neurons in the ventral gray matter. LY = LY-294002; PD = PD-98059; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion). * $P < 0.05$ *versus* control group.

pressions of both phospho-Akt and phospho-ERK in the spinal cord.

Timely restoration of blood flow is believed to be the most effective treatment to salvage neural tissue from prolonged ischemia. However, there is convincing evidence that sudden and full restoration of blood flow to ischemic cerebral tissue may paradoxically exaggerate injury that is not present at the end of ischemia. In clinical trials, patients treated with thrombolytic therapy have shown a 6% rate of intracerebral hemorrhage, which was balanced against a 30% improvement in functional outcome over controls. Destruction of the microvasculature and extension of the infarct area occur after cerebral reperfusion.[26,27] Ischemic postconditioning initially referred to a stuttering reperfusion performed immediately after reperfusion, for preventing reperfusion injury in myocardium. Thereafter, collective studies provided insights into the protective effects of postconditioning on cerebral ischemia. In rat models of focal cerebral ischemia, ischemic postconditioning was found to reduce infarct size[12–14] and inhibit neuronal apoptosis.[11] It has evolved into a concept that ischemic postconditioning may be a novel avenue to protect against brain injury after stroke.[28] In a rabbit model, we first reported the neuroprotective effects of postconditioning against ischemia–reperfusion injury of spinal cords.[15] The neuroprotective effects of ischemic postconditioning can be mainly attributed to reduction of reperfusion injury by the avoidance of sudden and full restoration of reperfusion. To attenuate the reperfusion injury, staged or controlled reperfusion with retarded restoration of full coronary blood flow or perfusion pressure has been proposed for many years.[29] Gradual reperfusion was demonstrated to induce neuroprotection on ischemic brain.[12] Controlled low-pressure perfusion was tested in our previous work and was found to protect ischemic spinal cords.[16] Just as the standard ischemic postconditioning, the protective intervention of this study was also performed at the beginning of reperfusion to avoid the sudden and full restoration of blood supply; therefore, we regarded this method as modified ischemic postconditioning. In a study by Bopassa *et al.*,[30] low-pressure perfusion was confirmed to generate cardioprotection as robust as that of postconditioning. However, it is not

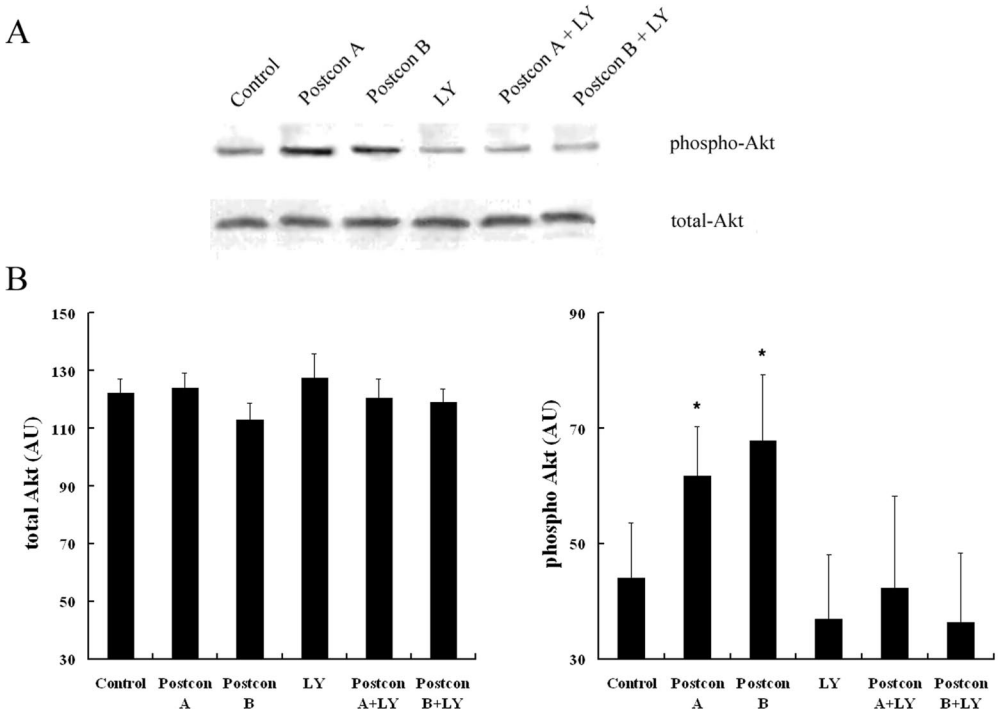

Fig. 4. Expressions of total Akt and phospho-Akt in the spinal cord. (*A*) Representative Western blot picture. (*B*) Densitometric analysis results expressed in arbitrary units (AU). LY = LY-294002; PD = PD-98059; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion). * $P < 0.05$ *versus* control group.

known whether low-pressure perfusion, modified postconditioning, is also as powerful as standard postconditioning to protect ischemic spinal cords. Our previous results showed that four to six cycles of 1-min occlusion and 1-min reperfusion applied at the just beginning of reperfusion were neuroprotective against spinal cord injury in rabbits.[15] In the current study, five cycles of 1-min occlusion and 1-min reperfusion was chosen, and

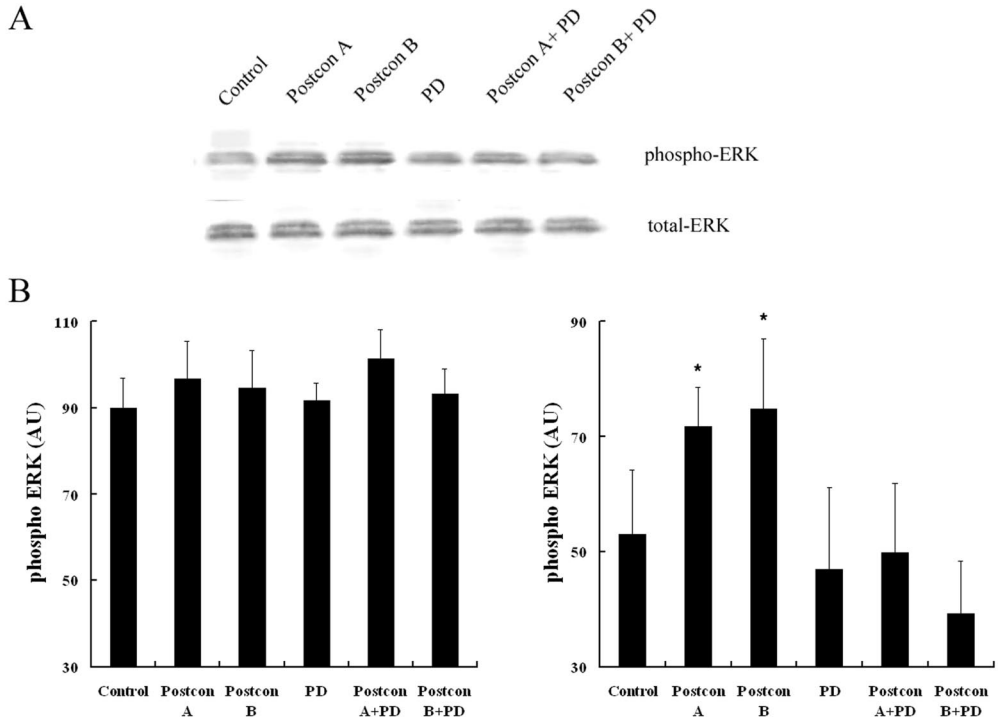

Fig. 5. Expressions of total extracellular signal–regulated kinase (ERK) and phospho-ERK in the spinal cord. (*A*) Representative Western blot picture. (*B*) Densitometric analysis results expressed in arbitrary units (AU). LY = LY-294002; PD = PD-98059; Postcon A = standard postconditioning; Postcon B = modified postconditioning (low-pressure reperfusion). * $P < 0.05$ *versus* control group.

the total period of postconditioning was 10 min. Hence, the two postconditioning methods were applied in the same model, and the total intervention times were equivalent. As expected, similar neuroprotective effects were detected as evidenced by the improvement of motor function and reduction of histologic damage in spinal cords. The results indicate that controlled low-pressure perfusion at the beginning of reperfusion, modified postconditioning, possesses neuroprotective effects against ischemia–reperfusion injury of the spinal cord comparable to that of standard postconditioning. Unlike standard postconditioning, the modified method can be performed without additional ischemia, which may be more acceptable for clinicians.

Although the current study has not uncovered the full mechanisms of the neuroprotective effects of postconditioning, some elements, including PI3K–Akt and ERK, have been identified in the signal transduction pathway. Several studies have confirmed that postconditioning protected the ischemic heart by activating the PI3K–Akt and/or ERK cascades.[10,17] In subsequent studies, the role of the P13K–Akt pathway has received major attention in different models of brain ischemic postconditioning. *In vivo* and *in vitro* data demonstrated that the infarction-reducing effects of postconditioning were abrogated in the presence of PI3K inhibitor LY294002.[13] In another rat model of focal cerebral ischemia, PI3K was detected to contribute to the long-term protection of postconditioning against stroke in rats.[12] Beneficial effects of ischemic postconditioning on global cerebral ischemia and reperfusion-induced behavioral deficits in mice were also confirmed to be mediated by activation of PI3K pathway.[18] In the current study, the PI3K–Akt pathway was activated, as the Akt was markedly phosphorylated by the two postconditioning methods. This phosphorylation, commensurate with the functional and structural protection, was abrogated by administration of PI3K inhibitor LY294002. Hence, our data suggest that the PI3K–Akt pathway is the predominant mediator of the neuroprotection induced by both standard and modified postconditioning.

However, while the role played by phosphorylated Akt in mediating neuroprotection has been sufficiently elucidated in different models of cerebral ischemia as well as spinal cord ischemia reported in the current study, contrasting results have been obtained with phosphorylated ERK. ERK activation has been shown to be involved in the mechanism of ischemic tolerance in the rat hippocampus[19] and the neuroprotection induced by ischemic preconditioning,[13] whereas in some other studies, inhibition of ERK has been indicated to be crucial in mediating neuroprotection induced by some neuroprotective agents.[31] ERK also contributed to neural cell death in the animal models of ischemia- and trauma-induced brain injuries.[32] Still now, little is known about the function of ERK in the neuroprotection of postconditioning. In the current study, the two postconditioning strategies markedly enhanced the expression of phospho-ERK in the spinal cord, and their neuroprotection was abrogated with intrathecal injection of ERK-selective antagonist, suggesting that the ERK pathway is required for sustained spinal cord protection of postconditioning.

Therefore, our pharmacologic studies reveal that the spinal cord protection observed in the current study is mediated by both PI3K and ERK pathways. Expressions of both PI3K and ERK were shown to prolong phosphorylation in the cortex of posctconditioned rats; however, different from our results, the neuroprotection after postconditioning was inhibited only in the presence of LY294002, which blocks Akt activation, but not U0126, the ERK inhibitor.[13] In accord with our results, it has been reported that PI3K combined with ERK mediated the neuroprotective effects afforded by ischemic preconditioning,[13] MCI-186,[33] brain-derived neurotrophic factor,[34] erythropoietin,[35] and postischemic administration of orthovanadate.[36] In myocardial protection generated by postconditioning, the activation of PI3K and/or ERK may converge on the mitochondrial permeability transition pore.[37] Evidence obtained over the past two decades shows that reactive oxygen species are involved in brain lesions due to cerebral ischemia–reperfusion. The mitochondria are the primary intracellular source of reactive oxygen species, because they generate huge numbers of oxidative-reduction reactions and use massive amounts of oxygen.[38] Therefore, the spinal cord protection of postconditioning may also be mediated by inhibition of the mitochondrial permeability transition pore through the activation of PI3K and ERK. However, further studies are required to delineate the downstream factors of PI3K and ERK in the postconditioning-mediated protection on the spinal cord.

In conclusion, the current study provides novel insights into the spinal cord protection induced by standard postconditioning, a series of brief mechanical interruptions of reperfusion at onset of reperfusion, and modified postconditioning, partial restoration of blood flow by controlled low-pressure perfusion at the beginning of reperfusion. Our findings demonstrate for the first time that the two postconditioning methods induce neuroprotective effects on the spinal cord to a comparable extent and share a common molecular mechanism, in which PI3K and ERK pathways play crucial roles. As a very simple procedure, postconditioning afforded by the two methods possesses a potential clinical value in the prevention of neurologic injury after thoracic aneurysm surgery.

References

1. Coselli JS, LeMaire SA, Conklin LD, Köksoy C, Schmittling ZC: Morbidity and mortality after extent II thoracoabdominal aortic aneurysm repair. Ann Thorac Surg 2002; 73:1107–15

2. Safi HJ, Miller CC III, Huynh TT, Estrera AL, Porat EE, Winnerkvist AN, Allen BS, Hassoun HT, Moore FA: Distal aortic perfusion and cerebrospinal fluid

drainage for thoracoabdominal and descending thoracic aortic repair: Ten years of organ protection. Ann Surg 2003; 238:372–80

3. Crawford ES, Crawford JL, Safi HJ, Coselli JS, Hess KR, Brooks B, Norton HJ, Glaeser DH: Thoracoabdominal aortic aneurysms: Preoperative and intraoperative factors determining immediate and long-term results of operations in 605 patients. J Vasc Surg 1986; 3:389–404

4. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36

5. Zvara DA, Colonna DM, Deal DD, Vernon JC, Gowda M, Lundell JC: Ischemic preconditioning reduces neurologic injury in a rat model of spinal cord ischemia. Ann Thorac Surg 1999; 68:874–80

6. Toumpoulis IK, Papakostas JC, Matsagas MI, Malamou-Mitsi VD, Pappa LS, Drossos GE, Derose JJ, Anagnostopoulos CE: Superiority of early relative to late ischemic preconditioning in spinal cord protection after descending thoracic aortic occlusion. J Thorac Cardiovasc Surg 2004; 128:724–30

7. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J: Inhibition of myocardial injury by ischemic postconditioning during reperfusion: Comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003; 285:H579–88

8. Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J: Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004; 62:74–85

9. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M: Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111:194–7

10. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM: Postconditioning: A form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004; 95:230–2

11. Xing B, Chen H, Zhang M, Zhao D, Jiang R, Liu X, Zhang S: Ischemic postconditioning inhibits apoptosis after focal cerebral ischemia/reperfusion injury in the rat. Stroke 2008; 39:2362–9

12. Gao X, Ren C, Zhao H: Protective effects of ischemic postconditioning compared with gradual reperfusion or preconditioning. J Neurosci Res 2008; 86:2505–11

13. Pignataro G, Meller R, Inoue K, Ordonez AN, Ashley MD, Xiong Z, Gala R, Simon RP: *In vivo* and *in vitro* characterization of a novel neuroprotective strategy for stroke: Ischemic postconditioning. J Cereb Blood Flow Metab 2008; 28:232–41

14. Zhao H, Sapolsky RM, Steinberg GK: Interrupting reperfusion as a stroke therapy: Ischemic postconditioning reduces infarct size after focal ischemia in rats. J Cereb Blood Flow Metab 2006; 26:1114–21

15. Jiang X, Shi E, Nakajima Y, Sato S: Postconditioning, a series of brief interruptions of early reperfusion, prevents neurologic injury after spinal cord ischemia. Ann Surg 2006; 244:148–53

16. Shi E, Jiang X, Kazui T, Washiyama N, Yamashita K, Terada H, Bashar AH: Controlled low-pressure perfusion at the beginning of reperfusion attenuates neurologic injury after spinal cord ischemia. J Thorac Cardiovasc Surg 2007; 133:942–8

17. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV: Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004; 44:1103–10

18. Rehni AK, Singh N: Role of phosphoinositide 3-kinase in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked behavioral deficits in mice. Pharmacol Rep 2007; 59:192–8

19. Choi JS, Kim HY, Cha JH, Lee MY: Ischemic preconditioning-induced activation of ERK1/2 in the rat hippocampus. Neurosci Lett 2006; 409:187–91

20. Zuo Z, Wang Y, Huang Y: Isoflurane preconditioning protects human neuroblastoma SH-SY5Y cells against *in vitro* simulated ischemia-reperfusion through the activation of extracellular signal-regulated kinases pathway. Eur J Pharmacol 2006; 542:84–91

21. Suzuki K, Kazui T, Terada H, Umemura K, Ikeda Y, Bashar AH, Yamashita K, Washiyama N, Suzuki T, Ohkura K, Yasuike J: Experimental study on the protective effects of edaravone against ischemic spinal cord injury. J Thorac Cardiovasc Surg 2005; 130:1586–92

22. Shi E, Kazui T, Jiang X, Washiyama N, Yamashita K, Terada H, Bashar AH: Intrathecal injection of bone marrow stromal cells attenuates neurologic injury after spinal cord ischemia. Ann Thorac Surg 2006; 81:2227–33

23. Tarlov IM: Spinal Cord Compression: Mechanisms of Paralysis and Treatment. Springfield, Illinois, Charles C. Thomas, 1957, p 147

24. Mutch WA, Graham MR, Halliday WC, Thiessen DB, Girling LG: Use of neuroanesthesia adjuncts (hyperventilation and mannitol administration) improves neurological outcome after thoracic aortic cross-clamping in dogs. Stroke 1993; 24:1204–10

25. Yu F, Narasimhan P, Saito A, Liu J, Chan PH: Increased expression of a proline-rich Akt substrate (PRAS40) in human copper/zinc-superoxide dismutase transgenic rats protects motor neurons from death after spinal cord injury. J Cereb Blood Flow Metab 2008; 28:44–52

26. Jean WC, Spellman SR, Nussbaum ES, Low WC: Reperfusion injury after focal cerebral ischemia: The role of inflammation and the therapeutic horizon. Neurosurgery 1998; 43:1382–96

27. Hossmann KA: Reperfusion of the brain after global ischemia: Hemodynamic disturbances. Shock 1997; 8:95–101

28. Zhao H: Ischemic postconditioning as a novel avenue to protect against brain injury after stroke. J Cereb Blood Flow Metab 2009; 29:873–85

29. Okamoto F, Allen BS, Buckberg GD, Bugyi H, Leaf J: Reperfusion conditions: Importance of ensuring gentle *versus* sudden reperfusion during relief of coronary occlusion. J Thorac Cardiovasc Surg 1986; 92:613–20

30. Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M: PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. Cardiovasc Res 2006; 69:178–85

31. Xu X, Chua CC, Gao J, Hamdy RC, Chua BH: Humanin is a novel neuroprotective agent against stroke. Stroke 2006; 37:2613–9

32. Zhuang S, Schnellmann RG: A death-promoting role for extracellular signal-regulated kinase. J Pharmacol Exp Ther 2006; 319:991–7

33. Niyaz M, Numakawa T, Matsuki Y, Kumamaru E, Adachi N, Kitazawa H, Kunugi H, Kudo M: MCI-186 prevents brain tissue from neuronal damage in cerebral infarction through the activation of intracellular signaling. J Neurosci Res 2007; 85:2933–42

34. Sun X, Zhou H, Luo X, Li S, Yu D, Hua J, Mu D, Mao M: Neuroprotection of brain-derived neurotrophic factor against hypoxic injury *in vitro* requires activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. Int J Dev Neurosci 2008; 26:363–70

35. Kilic E, Kilic U, Soliz J, Bassetti CL, Gassmann M, Hermann DM: Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. FASEB J 2005; 19:2026–8

36. Hasegawa Y, Hamada J, Morioka M, Yano S, Kawano T, Kai Y, Fukunaga K, Ushio Y: Neuroprotective effect of postischemic administration of sodium orthovanadate in rats with transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 2003; 23:1040–51

37. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ: Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004; 113:1535–49

38. Christophe M, Nicolas S: Mitochondria: A target for neuroprotective interventions in cerebral ischemia-reperfusion. Curr Drug Targets 2005; 6:821–33