

Protective effects of remote ischemic preconditioning against spinal cord ischemia–reperfusion injury in rats

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ANOVA, analysis of variance; CSF, cerebrospinal fluid; Glu, glutamate; d-ROMs, diacron-reactive oxygen metabolites; IPC, ischemic preconditioning IRI, ischemia–reperfusion injury; MEP, motor-evoked potential; MDI, motor deficit index; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NR2B, N-methyl-D-aspartate receptor 2B subunit; RIPC, remote ischemic preconditioning; SCI, spinal cord ischemia;

Central Message:

Remote ischemic preconditioning exhibited a protective effect against spinal cord ischemia probably due to the reduction of glutamate release and N-methyl-D-aspartate receptor 2B subunit expression.

Perspective Statement:

The excessive elevations of extracellular glutamate and NMDA receptor activation can lead to Ca^{2+} homeostasis disorder, which induce the activation of several signal pathways, and are associated with neuronal cell death. The current study showed that remote ischemic preconditioning prevented the increases in glutamate concentration and expression of NMDA receptor 2B subunit induced by spinal cord ischemia.

ABSTRACT

Objectives: We aimed to investigate the protective effect of remote ischemic preconditioning against spinal cord ischemia and find out a clue to its mechanism by measuring glutamate concentrations in the spinal ventral horn.

Methods: Male Sprague-Dawley rats were divided into five groups (n = 6 in each group) as follows: sham; SCI (only spinal cord ischemia); RPC/SCI (perform remote ischemic preconditioning before spinal cord ischemia); MK-801/RPC/SCI [administer MK-801, (*N-methyl-D-aspartate* [NMDA] receptor antagonist), before remote ischemic preconditioning]; and MK-801/SCI (administer MK-801 without remote ischemic preconditioning). Remote ischemic preconditioning was achieved by brief limb ischemia 80 min before spinal cord ischemia. MK-801 (1 mg/kg, intravenous) was administered 60 min before remote ischemic preconditioning. The glutamate concentration in the ventral horn was measured by microdialysis for 130 min following spinal cord ischemia. Immunofluorescence was also performed to evaluate the expression of NMDA receptor 2B subunit (NR2B) in the ventral horn 130 min after spinal cord ischemia.

Results: The glutamate concentrations in the SCI group were significantly higher than those in the sham group at all time points ($p < 0.01$). Remote ischemic preconditioning attenuated

the spinal cord ischemia-induced glutamate increase. When MK-801 was pre-administered before remote ischemic preconditioning, glutamate concentration was increased after spinal cord ischemia ($p<0.01$). Immunofluorescence showed that remote ischemic preconditioning prevented the increase in the expression of NR2B on the surface of motor neurons ($p=0.047$).

Conclusions: Our results showed that remote ischemic preconditioning prevented spinal cord ischemia-induced extracellular glutamate increase in ventral horn, and also suppressed NR2B expression.

Introduction

Paraparesis and paraplegia due to ischemia–reperfusion injury (IRI) in the spinal cord are among the most serious complications of thoracoabdominal aortic surgery. Postoperative spinal cord complications are associated with an increased incidence of early and late mortality,¹ and, therefore, protective interventions against IRI in the spinal cord are required. Over recent decades, various strategies have been developed, which have improved outcomes regarding postoperative IRI in the spinal cord. They are roughly divided into two categories: maintenance of circulation in the spinal cord during aortic cross-clamping, which includes cerebrospinal fluid (CSF) drainage,² and neuroprotection for the duration of ischemia–reperfusion, which includes intraoperative hypothermia³ and pharmacologic administration. However, the incidence of paraparesis and paraplegia after thoracoabdominal aortic surgery still remains high, ranging from 2.7–5.3%.⁴

Remote ischemic preconditioning (RIPC) has been proposed for neuroprotection over the duration of ischemia–reperfusion.⁵ Ischemic preconditioning (IPC) is a strategy for increasing ischemic tolerance by inducing nonlethal ischemic conditions before lethal ischemia. IPC induced by ischemia in the limbs or other organs is termed RIPC, and the protective effects of this noninvasive technique on the spinal cord have recently been

reported.⁵⁻⁷ However, the mechanism, which involves various biochemical messengers and activation of neuronal pathways, is still under investigation.⁷

We aimed to investigate the protective effect of RIPC against IRI in the spinal cord and reveal its mechanism by measuring glutamate concentrations in the ventral horn in the Sprague-Dawley rats using an *in vivo* microdialysis method. Previous experimental studies have confirmed the elevation of extracellular glutamate concentrations in CSF after spinal cord ischemia (SCI) using a microdialysis method and also investigated therapeutic interventions against IRI.⁸⁻¹⁰ However, the increase of glutamate concentration in CSF due to SCI is slow and delayed. Therefore, in the current study, we performed direct microdialysis in the spinal ventral horn. Direct measurement in the ventral horn will help detecting acute changes in glutamate concentration, which could reveal the detailed time course of IRI and, consequently, the mechanism of RIPC in spinal cord protection.

Methods

Before the initiation of the experiment, the experimental protocol was approved by the Institutional Animal Care and Use Committee of our institution (No.15026, Chairperson: Prof. Tetsuo Arakawa). All animals received humane care in compliance with the *Guiding Principles for the Care and Use of Animals* recommended by the Physiological Society of Japan. This study consisted of four phases. Experiment 1 was designed to investigate the protective effect of RIPC against IRI 2 h after spinal cord ischemia–reperfusion using an *in vivo* microdialysis method. Motor-evoked potential (MEP) amplitude was also measured to assess motor functions. Experiment 2 was designed to evaluate neurologic and histopathologic outcomes 48 h after ischemia–reperfusion. Experiment 3 was designed to evaluate the expression of N-methyl-D-aspartate (NMDA) receptor 2B subunit (NR2B) and adenosine A₁ receptor in the ventral horn using immunofluorescence 2 h after ischemia–reperfusion. Experiment 4 was designed to measure free radicals in the blood and ventral horn by using diacron-reactive oxygen metabolites (d-ROMs) test. Detailed information in each procedure was depicted in Supplemental information.

Animals and Surgical Preparation

Nine- to 13- week-old male Sprague-Dawley rats, weighing 300–550 g (Clea Inc., Osaka, Japan) were purchased. All rats were housed in plastic cages (1 rat per cage) at the animal center of Osaka City University with 12-h dark/light cycles and had free access to water and food. The animal center was maintained at a temperature of 22°C and humidity of 55-60%. All procedures were performed during the daytime in the laboratory. After induction of general anesthesia, 8 mg/kg 0.25% bupivacaine was administered subcutaneously at the surgical sites for intra- and post-operative analgesia before the incision. Intra-arterial cannulation (PE-50: Becton, Dickinson and Company, Franklin Lakes, NJ) was performed in the left carotid and right femoral arteries for monitoring and adjusting blood pressure. Intravenous cannulation (SP31: NATSUME SEISAKUSHO Co. Ltd., Tokyo, Japan) was performed in the left internal jugular vein. Tracheotomy was performed, and a tracheal cannula (16-gauge intravenous cannula [Insyte-W; Becton, Dickinson and Company]) was inserted, and then mechanical ventilation was started.

Spinal Cord Ischemia

SCI was induced by direct clamping of the descending thoracic aorta. A fourth left intercostal incision was made, and the descending thoracic aorta was clamped using a surgical clip at a

level just distal to the left subclavian artery. During SCI, blood was drawn from the left carotid arterial line to maintain the proximal arterial pressure at 40 mmHg and distal arterial pressure at 10 mmHg. SCI was continued for 12 min, after which the descending thoracic aorta was de-clamped, and the withdrawn blood was returned into the animal.

Remote Ischemic Preconditioning

RIPC was induced as previously reported.¹¹ In brief, four cycles of remote ischemia in the right upper and lower extremities were induced for 5 min, followed by 5 min of reperfusion. A thin elastic tourniquet was tightly fastened around the upper third of the limb to stop the blood flow. The completion of limb ischemia was documented by the skin changing color to cyanosis, which returned to normal after reperfusion.

Experiment 1

In Vivo Spinal Microdialysis

For inserting the microdialysis probe, laminectomy was performed, and the dura mater was torn at the level of the 10th thoracic vertebra. The tip of the microdialysis probe (D-I-4-01; Eicom, Kyoto, Japan) was placed in the ventral horn at an angle of 15° to the vertical line, 1.2

mm to the lateral side of the center of the spinal cord, and at a depth of 1.4 mm (Supplemental Figure 1). The probe was perfused (flow rate: 2 μ L/min) with Ringer's solution (147.0 mM NaCl, 4.0 mM KCl, and 2.3 mM CaCl₂). After constant perfusion for 20 min, the perfusate was collected (10 min/cycle, 2 cycles) to determine the baseline concentration. After baseline measurement, the dialysate was collected at 10-min intervals for 130 min in each group.

Motor Evoked Potential

For measuring the MEP, a cathodic stimulation lead (OA215-098; UNIQUE MEDICAL Co. Ltd., Tokyo, Japan) was placed 4 mm lateral and 5 mm caudal from the bregma, and an anodal stimulation lead (OA215-090a; UNIQUE MEDICAL) was inserted into the tongue. A sensing lead (OA215-059; UNIQUE MEDICAL) was also placed in the left hind limb. A grounding needle (OA215-098; UNIQUE MEDICAL) was placed in the subcutaneous tissue of the lower back. Supplemental Figure 2 shows the typical patterns of MEP amplitude. The baseline peak-to-peak amplitude was measured before SCI and subsequently every 10 min for 130 min in each group.

Protocol for Experiment 1

The protocols for Experiment 1 are summarized in Figure 1A. The rats were randomly divided into five groups (n=6 in each group): sham group (sham-operated); SCI group (only SCI); RIPC/SCI group (perform RIPC before SCI); MK-801/RIPC/SCI group (administer MK-801, an NMDA receptor antagonist, before RIPC); and MK-801/SCI group (administer MK-801 without RIPC). In the MK-801/RIPC/SCI and MK-801/SCI groups, to assess the association of the NMDA receptor with the mechanism of spinal cord protection by RIPC, MK-801 (Wako Pure Chemical Industries, Ltd., Osaka Japan) was administered intravenously (1 mg/kg) 140 min before aortic clamping. In the RIPC/SCI and MK-801/RIPC/SCI groups, RIPC was induced 80 min before aortic clamping. In each group, a microdialysis probe was inserted 40 min before aortic clamping. After baseline measurement, SCI was induced.

Experiment 2

Motor Function

Motor function was evaluated on the basis of ambulation and placing/stepping reflex in the hindlimb, as previously reported.¹² The detailed information was shown in the Supplemental information. For each rat, a motor deficit index (MDI) score, the sum of ambulation and

reflex scores, was calculated 48 h after reperfusion.

Histopathologic Evaluation

After physiologic evaluation, all rats were euthanized. The spinal cord was removed and preserved in formaldehyde for 72 h. Then, the segment of 10th thoracic spinal cord was dissected, embedded in paraffin, and sectioned at a thickness of 3 μ m. The slides were stained with Nissl stain. For histopathologic evaluation, the numbers of normal and dark-stained motor neurons in the ventral horn were counted. Intra-observer analysis was performed by using intraclass correlation coefficient (ICC) and Bland-Altman analysis.

Protocol for Experiment 2

The protocols for Experiment 2 are summarized in Figure 1B. As with Experiment 1, the rats were randomly divided into five groups. Following aortic clamping for 12 min, the chest was closed. All catheters and the tracheostomy tube were removed, and the wounds were sutured. Following recovery from anesthesia, all rats were returned to the cages. Physiologic and histopathologic evaluation was performed 48 h after ischemia–reperfusion.

Experiment 3

Immunofluorescence

In order to examine the association of NR2B subunit and adenosine A₁ receptor with the mechanisms of SCI and RIPC, additional 18 rats (Sham, SCI and RIPC/SCI group) were euthanized after 130 min from SCI. Spinal cord extraction was performed, and the sections were incubated with primary antibody of anti-NR2B (ab65783, 1:200; Abcam, Cambridge, UK) and anti-adenosine A₁ receptor (ab82477, 1:100; Abcam) for 60 min at room temperature. Then, the sections were incubated with secondary antibody (Alexa Fluor 488, 1:200; Abcam) for 60 min at room temperature. The middle of ventral horn was observed, and the brightest motor neuron was picked up. In order to quantify the receptor expression on the surface of motor neuron, the ratio of the mean fluorescence intensity of both sides of the motor neuron to the intraneuronal minimal fluorescence intensity (the ratio of S/C) was calculated.¹³ The protocols for Experiment 3 are summarized in Figure 1C.

Experiment 4

Oxidative stress evaluation

To evaluate oxidative stress, the amount of hydroperoxides in blood and spinal cord perfusate

was measured with the diacron-reactive oxygen metabolites (d-ROMs) test. The detailed information of d-ROMs test was shown in the Supplemental information. Additional 18 rats (Sham, SCI and RIPC/SCI group) were anesthetized and underwent surgical procedures (microdialysis in the ventral horn) as Experiment 1. Blood and microdialysis perfusate were collected (30 min/cycle, 2 cycles) to determine the baseline. After baseline measurement, blood and perfusate were collected at 30-min intervals for 120 min in each group. The protocols for Experiment 4 are summarized in Figure 1D.

Statistical Analysis

The sample size of each group was referred to the previous study investigating the protective effect of RIPC against SCI.⁶ The glutamate concentrations, MEP amplitudes, and the amount of hydroperoxides measured before aortic clamping were considered as the 100% basal values. These variables were expressed as the percentage change from the basal value at each time point and analyzed by two-way repeated-measures analysis of variance (ANOVA), followed by the Tukey post-hoc analysis. Before performing two-way repeated-measures ANOVA, the glutamate concentration, MEP amplitude data were subjected to log transformation. MDI scores, numbers of neurons, and the ratio of S/C were analyzed by

one-way ANOVA, followed by the Tukey post-hoc test. All variables were expressed as median and interquartile range. Statistical analyses were performed using StatFlex version 6.0 (Artech. Co., Ltd., Osaka, Japan); $p < 0.05$ was considered statistically significant.

Results

Glutamate Concentrations

The glutamate concentrations in the SCI group were significantly elevated relative to the baseline values ($p<0.01$) and were significantly higher than those in the sham group at all time points ($p<0.01$); in contrast, the glutamate concentrations in the RIPC/SCI group were not significantly increased at any of the time points (Figure 2A). The glutamate concentrations in the MK-801/RIPC/SCI and MK-801/SCI groups were significantly elevated at all time points relative to their respective baseline values ($p<0.05$) and were significantly higher than those in the RIPC/SCI group ($p<0.05$) (Figure 2B).

MEP Amplitude

In the SCI group, the MEP amplitudes were significantly decreased relative to the baseline values after SCI ($p<0.01$) and were significantly lower than those in the sham group at all time points ($p<0.05$). In contrast, the MEP amplitudes in the RIPC/SCI group remained at above 50% of the baseline value and were significantly higher than those in the SCI group at all time points ($p<0.05$) (Figure 3A). The MEP amplitudes in the MK-801/RIPC/SCI and MK-801/SCI groups were significantly decreased relative to the baseline values at all time

points ($p<0.01$) and were significantly lower than those in the RIPC/SCI group ($p<0.01$) (Figure 3B).

Evaluation of Motor Function

The MDI scores in all groups were significantly higher than those in the sham group ($p<0.05$).

The MDI scores in the RIPC/SCI group were significantly lower than those in the SCI group ($p<0.001$). The MK-801/RIPC/SCI and MK-801/SCI groups had significantly higher MDI scores than the RIPC/SCI group ($p=0.043$ and $p=0.005$, respectively) (Figure 4).

Histopathologic Evaluation

In comparison to the sham group, the SCI group showed a significantly lower number of normal neurons and a significantly higher percentage of dark-stained neurons ($p<0.001$). The RIPC/SCI group had significantly more normal neurons and fewer dark-stained neurons than the SCI group ($p<0.001$). On the other hand, the MK-801/RIPC/SCI and MK-801/SCI groups exhibited significantly lower numbers of normal neurons and significantly higher percentages of dark-stained neurons than the RIPC/SCI group (number of normal neurons: $p<0.01$; percentage of dark-stained neurons: $p<0.001$; respectively) (Figure 5). Additionally,

intra-observer analysis represented a good reproducibility (Supplemental Table 1).

Immunofluorescence

The enhancement of membrane NR2B expression in the ventral horn was observed in the SCI group, while the expression was significantly decreased in the RIPC/SCI group. The ratio of S/C in the SCI group was significantly higher than that in the Sham group ($p=0.039$), while the ratio of S/C in the RIPC/SCI group was significantly lower than that in the SCI group ($p=0.047$) (Figure 6). Regarding adenosine A₁ receptor, there were no significant differences in the ratio of S/C among the groups ($p=0.560$) (Supplemental Figure 3).

Hydroperoxides assay

There were no significant differences in the amount of arterial blood hydroperoxides among the groups and any time points (Supplemental Figure 4A). In the spinal cord perfusate, the amount of hydroperoxides in the SCI group was significantly elevated compared with the baseline values ($p<0.01$) and was significantly higher than that in the sham group at each time point ($p<0.01$); in contrast, the amount of hydroperoxides in the RIPC/SCI group was not significantly increased at any of the time points (Supplemental Figure 4B).

Discussion

In the present study, markedly increased extracellular glutamate and membrane NR2B expression in the ventral horn were observed in SCI (Supplemental Figure 5). RIPC prevented an increase in glutamate concentration in the ventral horn, and improved the post-SCI hindlimb motor function and histopathologic changes in the spinal cord 48 h after reperfusion. Additionally, RIPC prevented the increase in the expression of NR2B on motor neurons induced by SCI. In contrast, even with RIPC, pre-administration of MK-801 led to an increase in glutamate concentration in the ventral horn, and worsening of neurologic outcomes. These results suggested that the increase in glutamate concentration and the excessive expression of NR2B in the ventral horn are related to spinal cord injury, and RIPC shows a protective effect against SCI by preventing these changes. Visual summary of the current study is depicted as Figure 7.

Glutamate plays an important role in the central nervous system. However, overaccumulation of glutamate after central nervous system insult leads to overstimulation of the NMDA receptor, followed by neuronal injury, called excitotoxicity.^{8,9,14} Excessive NMDA receptor activation can lead to Ca^{2+} homeostasis disorder, which induces the activation of several cytokines and proteases, thus causing free radical and nitric oxide (NO) production.¹⁵

Activation of these signal pathways is associated with neuronal cell death. Moreover, cytokines enhance calcium-dependent glutamate release, and this enhancement is mediated by NO.¹⁶ It has also been reported that glutamate induced overexpression of NMDA receptor following cerebral ischemia.^{17,18} The mRNA expression of NMDA receptor increased after cerebral ischemia, and glutamate itself plays a key role in the induction of NMDA receptor subunit expression. Furthermore, this inductive effect of glutamate occurs mainly via α -amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA) receptor.¹⁷ In the present study, SCI markedly increased extracellular glutamate and membrane NR2B expression in the ventral horn, which could lead to excessive NMDA receptor activation and may eventually develop spinal cord injury.

In the current study, RIPC attenuated the increase in glutamate concentration following SCI probably by preventing excessive NMDA receptor activation. RIPC also suppressed the elevation of hydroperoxides in the spinal cord after SCI, which might reflect the modulation of the NMDA receptor-mediated signaling pathways. Recent studies have shown protective effects of RIPC on the spinal cord.^{5-7,19} Dong et al.⁶ studied the impact of RIPC on SCI in a rabbit model and found that RIPC improved the neurologic outcomes. They also showed that dimethylthiourea, a free radical scavenger, inhibits the preventive effects of

RIPC on SCI and revealed that RIPC-induced pre-oxidative stress plays an important role in conferring tolerance against SCI. RIPC also showed spinal cord protection on porcine models.^{5,7} In these studies, the MEP amplitude increased significantly in the RIPC groups. Fukui et al.¹⁹ compared the impact of direct IPC, kidney RIPC, and limb RIPC on motor function, and concluded that only the direct IPC could decrease spinal cord injury. Haapanen et al.²⁰ reported that RIPC did not show an antioxidative effect at 24 hours after SCI. In these previous studies, the former results were consistent with our results, while the latter were not. This discrepancy might be due to differences in experimental settings, including the type of animals, and the methods of aortic occlusion and RIPC.

Our results also indicated that pre-administration of an NMDA receptor antagonist counteracts RIPC effects, whereas NMDA receptor antagonist without RIPC did not attenuate the increase in glutamate concentration. NMDA receptor antagonist itself is considered harmless to neuronal cells, and has a protective effect against ischemia-perfusion injury in the spinal cord.²¹ However, previous studies also showed that NMDA receptor antagonist interferes with the effect of NMDA-induced preconditioning, when administered before preconditioning.^{22,23} NMDA receptors have already been proven to be involved in the mechanism of brain protection by IPC.²⁴⁻²⁶ Sublethal ischemia is associated with a mild

increase in glutamate release by the affected cells,²⁷ leading to sublethal activation of NMDA receptors, resulting in the activation of several signal transduction pathways.^{26,28} Therefore, RIPC would lead to preconditioning of NMDA receptors. The results of immunofluorescence support this hypothesis because RIPC prevented the increase in the expression of NR2B on motor neurons induced by SCI.

Previous studies reported that a number of the mechanisms including humoral and neural pathways may be involved in the mechanism of RIPC.^{29,30} Dong et al. previously reported that humoral factors, rather than the neural pathway, mediate the ischemic tolerance of RIPC in SCI.⁶ Adenosine is one of the well-studied humoral factors. We examined the expression of adenosine A₁ receptor after SCI. However, the expression of adenosine A₁ receptor was not changed among the groups. Similar to our findings, Constantino et al. reported that NMDA preconditioning did not affect the expression of adenosine A₁ receptor.³¹ Considering these results including ours, adenosine A₁ receptor may not be involved in the protective mechanism of RIPC against SCI. However, as previously reported,^{29,30} the mechanism of RIPC for spinal cord protection may be multifactorial, and should be further investigated.

Our study has some methodological limitations. First, we used sevoflurane in

Experiment 2. Some studies have reported that sevoflurane preconditioning has a neuroprotective effect.^{32,33} Therefore, the results of Experiment 2 might have been influenced by sevoflurane. However, the concentration of sevoflurane used in the Experiment 2 was fixed (5% until tracheotomy and 3% after tracheotomy), and all experimental procedures were performed in the same way. Therefore, the amount of sevoflurane was similar among the groups, and the impact of sevoflurane on the results was minimized. Second, some other parameters including glucose, lactate, pyruvate, and glycerol can be measured using microdialysis. These molecules are useful to assess metabolic state after spinal cord injury,^{34,35} however, cannot be measured in our experimental settings. Third, the glutamate concentration, MEP amplitude, and the amount of hydroperoxides were analyzed as the percentage changes from the baseline. Comparison using absolute values may be preferable, however, these variables markedly vary among individuals. Therefore, in this study, we used the percentage changes from the baseline to analyze these data, as previously performed.^{5,8,34} Furthermore, the changes of glutamate concentration and NR2B expression were observed in a rodent model, however, may not be applicable to humans undergoing aneurysm surgery. Further studies are needed to investigate whether RIPC exhibits a protective effect against SCI even in humans.

In conclusion, direct microdialysis in the spinal ventral horn detected marked increase in glutamate concentration, and the enhancement of membrane NR2B expression in the ventral horn were observed after SCI. Both of which might lead excessive NMDA receptor activation and exacerbate ischemic spinal cord injury. RIPc exhibited a protective effect against SCI probably in part due to the reduction of glutamate release and NR2B expression.

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References

1. Shimizu H, Yozu R. Current strategies for spinal cord protection during thoracic and thoracoabdominal aortic aneurysm repair. *Gen Thorac Cardiovasc Surg*. 2011;59:155-63.
2. Coselli JS, LeMaire SA, Koksoy C, Schmittling ZC, Curling PE. Cerebrospinal fluid drainage reduces paraplegia after thoracoabdominal aortic aneurysm repair: results of a randomized clinical trial. *J Vasc Surg*. 2002;35:631-9.
3. Svensson LG, Khitin L, Nadolny EM, Kimmel WA. Systemic temperature and paralysis after thoracoabdominal and descending aortic operations. *Arch Surg*. 2003;138:175-80.
4. Sloan TB, Edmonds HL, Jr., Koht A. Intraoperative electrophysiologic monitoring in aortic surgery. *J Cardiothorac Vasc Anesth*. 2013;27:1364-73.
5. Haapanen H, Herajarvi J, Arvola O, Anttila T, Starck T, Kallio M, et al. Remote ischemic preconditioning protects the spinal cord against ischemic insult: An experimental study in a porcine model. *J Thorac Cardiovasc Surg*. 2016;151:777-85.
6. Dong HL, Zhang Y, Su BX, Zhu ZH, Gu QH, Sang HF, et al. Limb remote ischemic preconditioning protects the spinal cord from ischemia-reperfusion injury: a newly identified nonneuronal but reactive oxygen species-dependent pathway. *Anesthesiology*. 2010;112:881-91.
7. Herajarvi J, Anttila T, Sarja H, Mustonen C, Haapanen H, Makela T, et al. Exploring

Spinal Cord Protection by Remote Ischemic Preconditioning: An Experimental Study. *Ann Thorac Surg.* 2017;103:804-811.

8. Hsieh YC, Cheng H, Chan KH, Chang WK, Liu TM, Wong CS. Protective effect of intrathecal ketorolac in spinal cord ischemia in rats: a microdialysis study. *Acta Anaesthesiol Scand.* 2007;51:410-4.

9. Kakinohana M, Kakinohana O, Jun JH, Marsala M, Davison KJ, Sugahara K. The activation of spinal N-methyl-D-aspartate receptors may contribute to degeneration of spinal motor neurons induced by neuraxial morphine after a noninjurious interval of spinal cord ischemia. *Anesth Analg.* 2005;100:327-34.

10. Jellish WS, Zhang X, Langen KE, Spector MS, Scalfani MT, White FA. Intrathecal magnesium sulfate administration at the time of experimental ischemia improves neurological functioning by reducing acute and delayed loss of motor neurons in the spinal cord. *Anesthesiology.* 2008;108:78-86.

11. Xu J, Sun S, Lu X, Hu X, Yang M, Tang W. Remote ischemic pre- and postconditioning improve postresuscitation myocardial and cerebral function in a rat model of cardiac arrest and resuscitation. *Crit Care Med.* 2015;43:e12-8.

12. Taira Y, Marsala M. Effect of proximal arterial perfusion pressure on function, spinal

cord blood flow, and histopathologic changes after increasing intervals of aortic occlusion in the rat. *Stroke*. 1996;27:1850-8.

13. Aguetaz E, Lopez JJ, Krzesiak A, Lipskaia L, Adnot S, Hajjar RJ, et al. Axial stretch-dependent cation entry in dystrophic cardiomyopathy: Involvement of several TRPs channels. *Cell Calcium*. 2016;59:145-155.

14. Rokkas CK, Helfrich LR, Jr., Lobner DC, Choi DW, Kouchoukos NT. Dextrorphan inhibits the release of excitatory amino acids during spinal cord ischemia. *Ann Thorac Surg*. 1994;58:312-20.

15. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. *Cell Calcium*. 2010;47:122-9.

16. Ida T, Hara M, Nakamura Y, Kozaki S, Tsunoda S, Ihara H. Cytokine-induced enhancement of calcium-dependent glutamate release from astrocytes mediated by nitric oxide. *Neurosci Lett*. 2008;432:232-6.

17. Heurteaux C, Lauritzen I, Widmann C, Lazdunski M. Glutamate-induced overexpression of NMDA receptor messenger RNAs and protein triggered by activation of AMPA/kainate receptors in rat hippocampus following forebrain ischemia. *Brain Res*. 1994;659:67-74.

18. Zhao P, Yang JM, Wang YS, Hao YJ, Li YX, Li N, et al. Neuroprotection of Cytisine

Against Cerebral Ischemia-Reperfusion Injury in Mice by Regulating NR2B-ERK/CREB Signal Pathway. *Neurochem Res.* 2018;43:1575-1586.

19. Fukui T, Ishida K, Mizukami Y, Shiramoto K, Harada H, Yamashita A, et al. Comparison of the protective effects of direct ischemic preconditioning and remote ischemic preconditioning in a rabbit model of transient spinal cord ischemia. *J Anesth.* 2018;32:3-14.

20. Haapanen HJ, Herajarvi J, Honkanen HP, Mustonen C, Tuominen H, Puistola U, et al. Immunohistochemical Analysis of the Spinal Cord Ischemia- Effect of Remote Ischemic Preconditioning in a Porcine Model. *Heart Surg Forum.* 2018;21:E209-e214.

21. Martinez-Arizala A, Rigamonti DD, Long JB, Kraimer JM, Holaday JW. Effects of NMDA receptor antagonists following spinal ischemia in the rabbit. *Exp Neurol.* 1990;108:232-40.

22. Bond A, Lodge D, Hicks CA, Ward MA, O'Neill MJ. NMDA receptor antagonism, but not AMPA receptor antagonism attenuates induced ischaemic tolerance in the gerbil hippocampus. *Eur J Pharmacol.* 1999;380:91-9.

23. Rejdak R, Rejdak K, Sieklucka-Dziuba M, Stelmasiak Z, Grieb P. Brain tolerance and preconditioning. *Pol J Pharmacol.* 2001;53:73-9.

24. Liu XQ, Sheng R, Qin ZH. The neuroprotective mechanism of brain ischemic preconditioning. *Acta Pharmacol Sin.* 2009;30:1071-80.

25. Grabb MC, Choi DW. Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. *J Neurosci.* 1999;19:1657-62.
26. Sragovich S, Bromberg Y, Sperling O, Zoref-Shani E. Molecular alterations associated with the NMDA preconditioning-induced neuroprotective mechanism against glutamate cytotoxicity. *J Mol Neurosci.* 2012;47:519-32.
27. Dawson VL, Dawson TM. Mining for survival genes. *Biochem Soc Trans.* 2006;34:1307-9.
28. Navon H, Bromberg Y, Sperling O, Shani E. Neuroprotection by NMDA preconditioning against glutamate cytotoxicity is mediated through activation of ERK 1/2, inactivation of JNK, and by prevention of glutamate-induced CREB inactivation. *J Mol Neurosci.* 2012;46:100-8.
29. Lim SY, Hausenloy DJ. Remote ischemic conditioning: from bench to bedside. *Front Physiol.* 2012;3:27.
30. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res.* 2015;116:674-99.
31. Constantino LC, Pamplona FA, Matheus FC, Ludka FK, Gomez-Soler M, Ciruela F, et al. Adenosine A1 receptor activation modulates N-methyl-d-aspartate (NMDA) preconditioning phenotype in the brain. *Behav Brain Res.* 2015;282:103-10.
32. Codaccioni JL, Velly LJ, Moubarik C, Bruder NJ, Pisano PS, Guillet BA. Sevoflurane

preconditioning against focal cerebral ischemia: inhibition of apoptosis in the face of transient improvement of neurological outcome. *Anesthesiology*. 2009;110:1271-8.

33. Ding Q, Wang Q, Deng J, Gu Q, Hu S, Li Y, et al. Sevoflurane preconditioning induces rapid ischemic tolerance against spinal cord ischemia/reperfusion through activation of extracellular signal-regulated kinase in rabbits. *Anesth Analg*. 2009;109:1263-72.

34. Saether OD, Backstrom T, Aadahl P, Myhre HO, Norgren L, Ungerstedt U. Microdialysis of the spinal cord during thoracic aortic cross-clamping in a porcine model. *Spinal Cord*. 2000;38:153-7.

35. Chen S, Phang I, Zoumprouli A, Papadopoulos MC, Saadoun S. Metabolic profile of injured human spinal cord determined using surface microdialysis. *J Neurochem*. 2016;139:700-5.

Figure Legends

Figure 1. Protocols for (A) Experiment 1, (B) Experiment 2, (C) Experiment 3 and (D) Experiment 4. All rats were randomly divided into five groups: sham group (sham-operated); SCI group (only SCI); RIPC/SCI group (perform RIPC before SCI); MK-801/RIPC/SCI group (administer MK-801 before RIPC); and MK-801/SCI group (administer MK-801 without RIPC). Experiment 1 was designed to investigate the protective effect of RIPC 2 h after spinal cord ischemia–reperfusion using an *in vivo* microdialysis method. MEP amplitude was also measured to assess motor functions. Experiment 2 was designed to evaluate neurologic and histopathologic outcomes 48 h after ischemia–reperfusion. Experiment 3 was designed to evaluate the expression of N-methyl-D-aspartate (NMDA) receptor 2B subunit (NR2B) and adenosine A₁ receptor in the ventral horn using immunofluorescence 2 h after ischemia–reperfusion. Experiment 4 was designed to measure free radicals in the blood and ventral horn by using d-ROMs test.

Abbreviations: RIPC, remote ischemic preconditioning; SCI, spinal cord ischemia; MEP, motor-evoked potential; MDI, motor deficit index; d-ROMs, diacron-reactive oxygen metabolites.

Figure 2. Glutamate concentrations in the ventral horn in different treatment groups: (A) sham, SCI, and RIPC/SCI groups; (B) RIPC/SCI, MK-801/RIPC/SCI, and MK-801/SCI groups. The glutamate concentrations in the SCI group were significantly higher than those in the sham group. RIPC attenuated the spinal cord ischemia-induced glutamate increase (A). In contrast, even with RIPC, pre-administration of MK-801 (an NMDA receptor antagonist) led to an increase in glutamate concentration in the ventral horn (B). In each individual, one measurement was performed at each time interval. Values are expressed as percentage change from the baseline value at each time point. Data are shown as median and interquartile range. * $p < 0.05$ and ** $p < 0.01$, compared to the baseline value of each group.

Abbreviations: SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning; NMDA, N-methyl-D-aspartate.

Figure 3. Motor-evoked potential amplitudes in different treatment groups: (A) sham, SCI, and RIPC/SCI groups; (B) RIPC/SCI, MK-801/RIPC/SCI, and MK-801/SCI groups. In the SCI group, the MEP amplitudes were significantly lower than those in the sham group. In contrast, the MEP amplitudes in the RIPC/SCI group remained at above 50% of the baseline value and were significantly higher than those in the SCI group (A). Even with RIPC,

pre-administration of MK-801 (an NMDA receptor antagonist) led to a decrease in the MEP amplitude (B). In each individual, one measurement was performed at each time interval. Values are expressed as percentage change from the baseline value at each time point. Data are shown as median and interquartile range. * $p < 0.05$ and ** $p < 0.01$, compared to the baseline value of each group.

Abbreviations: SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning; MEP, motor-evoked potential; NMDA, N-methyl-D-aspartate.

Figure 4. MDI scores in all groups. Score 0 indicates completely normal motor function, whereas score 6 indicates complete paraplegia. RIPC improved the post-SCI hindlimb motor function 48 h after spinal cord ischemia–reperfusion. In contrast, even with RIPC, pre-administration of MK-801 (an NMDA receptor antagonist) led to worsening of hindlimb motor function. The upper and lower borders of the box represent the upper and lower quartiles. The middle horizontal line represents the median. The upper and lower whiskers represent the maximum and minimum values of non-outliers. * $p < 0.05$ versus the sham group; † $p < 0.05$ versus the SCI group; ‡ $p < 0.05$ versus the RIPC/SCI group; § $p < 0.05$ versus the MK-801/RIPC/SCI group.

Abbreviations: MDI, motor deficit index; RIPC, remote ischemic preconditioning; SCI, spinal cord ischemia; NMDA, N-methyl-D-aspartate.

Figure 5. Histopathologic evaluation. Representative photomicrographs of Nissl-stained sections of the ventral horn in the (A) sham and (B) SCI groups (at $\times 20$ magnification).

Dark-stained neurons (arrow) are observed in figure 5B. (C) Number of normal neurons and percentage of dark-stained neurons (as a percentage of total number of neurons) in each group. In comparison with the sham group, the SCI group showed a significantly lower number of normal neurons and a significantly higher percentage of dark-stained neurons.

RIPC improved the histopathologic changes in the spinal cord 48 h after spinal cord ischemia–reperfusion. In contrast, even with RIPC, pre-administration of MK-801 (an NMDA receptor antagonist) led to worsening of neurologic outcomes. The upper and lower borders of the box represent the upper and lower quartiles. The middle horizontal line represents the median. The upper and lower whiskers represent the maximum and minimum values of non-outliers. * $p < 0.05$ versus the sham group; $^{\dagger}p < 0.05$ versus the SCI group; $^{\ddagger}p < 0.05$ versus the RIPC/SCI; $^{\S}p < 0.05$ versus the MK-801/RIPC/SCI group.

Abbreviations: RIPC, remote ischemic preconditioning; SCI, spinal cord ischemia; NMDA,

N-methyl-D-aspartate.

Figure 6. NR2B expression in the ventral horn and motor neuron. Representative immunostaining images of NR2B in the spinal cord in the (A) sham, (B) SCI, and (C) RIPC/SCI groups (at $\times 4$ magnification). Box areas were observed at $\times 20$ magnification. (D) The pictures at the top of the panel D show immunostaining images in the Sham and SCI groups. The vertical red lines drawn in the immunostaining images represent the area selected for plotting a fluorescence profile. The jagged lines at the bottom show the fluorescence amplitude profile. In order to quantify the fluorescence intensity on the surface of motor neuron, the fluorescence intensities on the surface of motor neuron (S1 and S2) were measured, and the mean of these 2 values (S) was divided by the minimal fluorescence intensity value (C). (E) The ratios of S/C in the sham, SCI, and RIPC/SCI groups. Markedly increased NR2B expression in the ventral horn was observed in the SCI group. RIPC prevented the increase in the expression of NR2B on motor neurons induced by SCI. The upper and lower borders of the box represent the upper and lower quartiles. The middle horizontal line represents the median. The upper and lower whiskers represent the maximum and minimum values of non-outliers. * $p < 0.05$ versus the sham group; $^{\dagger}p < 0.05$ versus the

SCI group; $^{\ddagger}p < 0.05$ versus the RIPC/SCI group.

Abbreviations: NR2B, N-methyl-D-aspartate receptor 2B subunit; SCI, spinal cord ischemia;

RIPC, remote ischemic preconditioning; NMDA, N-methyl-D-aspartate.

Figure 7 (Graphical Abstract).

(Methods) Male Sprague-Dawley rats were divided into five groups ($n = 6$ in each group) as follows: sham; SCI (only spinal cord ischemia); RIPC/SCI (perform remote ischemic preconditioning before spinal cord ischemia); MK-801/RIPC/SCI [administer MK-801, (*N-methyl-D-aspartate* [NMDA] receptor antagonist), before remote ischemic preconditioning]; and MK-801/SCI (administer MK-801 without remote ischemic preconditioning). Spinal cord ischemia was induced by direct clamping of the descending thoracic aorta for 12 min. Remote ischemic preconditioning was induced by four cycles of remote ischemia in the right upper and lower extremities for 5 min, followed by 5 min of reperfusion. The glutamate concentration in the ventral horn was measured by microdialysis for 130 min following spinal cord ischemia. Immunofluorescence was also performed to evaluate the expression of NMDA receptor 2B subunit (NR2B) in the ventral horn 130 min after spinal cord ischemia.

(Results) The glutamate concentrations in the SCI group were significantly higher than those in the sham group. Remote ischemic preconditioning attenuated the spinal cord ischemia-induced glutamate increase. In contrast, even with remote ischemic preconditioning, pre-administration of MK-801 led to an increase in glutamate concentration in the ventral horn. The results of immunofluorescence showed that markedly increased NR2B expression in the ventral horn was observed in the SCI group. Remote ischemic preconditioning prevented the increase in the expression of NR2B on motor neurons induced by spinal cord ischemia.

(Implication) RIPC exhibited a protective effect against spinal cord ischemia probably in part due to the reduction of glutamate release and NR2B expression.

Abbreviations: SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning; NMDA, N-methyl-D-aspartate; NR2B, N-methyl-D-aspartate receptor 2B subunit.

Supplemental Figure 1. Position of the microdialysis probe in the ventral horn.

Low-magnification image ($\times 4$) to identify the tract of the microdialysis probe. The tip of the microdialysis probe was placed in the ventral horn at an angle of 15° to the vertical line, 1.2 mm to the lateral side of the center of the spinal cord, and at a depth of 1.4 mm. The tip of the probe is indicated by the arrow.

Supplemental Figure 2. Typical waveform patterns of motor-evoked potentials.

Peak-to-peak amplitudes were significantly decreased after spinal cord ischemia.

Supplemental Figure 3.

Adenosine A_1 receptor expression in the ventral horn and motor neuron. Representative immunostaining images of adenosine A_1 receptor in the spinal cord in the (A) sham, (B) SCI, and (C) RIPC/SCI groups (at $\times 4$ magnification). Box areas were observed at $\times 20$ magnification. (D) The ratios of S/C in the sham, SCI, and RIPC/SCI groups. There were no significant differences in the ratio of S/C among the groups. The upper and lower borders of the box represent the upper and lower quartiles. The middle horizontal line represents the median. The upper and lower whiskers represent the maximum and minimum values of

non-outliers.

Abbreviations: SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning.

Supplemental Figure 4. The amount of hydroperoxides in (A) blood and (B) spinal cord perfusate. In each individual, one measurement was performed at each time interval. The hydroperoxides are intermediate oxidative products of lipids, peptides and amino acids, and their amount is related to that of the free radicals. Two-way repeated-measures ANOVA showed that there were no significant differences in the amount of arterial blood hydroperoxides among the groups and any time points. In the spinal cord perfusate, the amount of hydroperoxides in the SCI group was significantly elevated compared with the baseline values ($p < 0.01$) and was significantly higher than that in the sham group at each time point ($p < 0.01$); in contrast, the amount of hydroperoxides in the RIPC/SCI group was not significantly increased at any of the time points.

Abbreviations: ANOVA, analysis of variance; SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning.

Supplemental Figure 5 (Central Picture). The results of glutamate measurement and

immunofluorescence in the ventral horn.

The glutamate concentrations in the SCI group were significantly higher than those in the sham group. Remote ischemic preconditioning attenuated the spinal cord ischemia-induced glutamate increase. In contrast, even with remote ischemic preconditioning, pre-administration of MK-801 led to an increase in glutamate concentration in the ventral horn. The results of immunofluorescence showed that markedly increased NR2B expression in the ventral horn was observed in the SCI group. remote ischemic preconditioning prevented the increase in the expression of NR2B on motor neurons induced by spinal cord ischemia.

Abbreviations: SCI, spinal cord ischemia; RIPC, remote ischemic preconditioning; NMDA, N-methyl-D-aspartate; NR2B, N-methyl-D-aspartate receptor 2B subunit.

Video Legend

After induction of anesthesia, intra-arterial cannulation was performed in the left carotid and right femoral arteries for monitoring and adjusting blood pressure. Intravenous cannulation was performed in the left internal jugular vein. Tracheotomy was performed, and a tracheal cannula was inserted.

For inserting the microdialysis probe, laminectomy was performed at the level of the 10th thoracic vertebra. For measuring the MEP, a cathodic stimulation lead was placed 4 mm lateral and 5 mm caudal from the bregma, and an anodal stimulation lead was inserted into the tongue. A sensing lead was also placed in the left hind limb. A grounding needle was placed in the subcutaneous tissue of the lower back.

SCI was induced by direct clamping of the descending thoracic aorta. A fourth left intercostal incision was made, and the descending thoracic aorta was clamped using a surgical clip at a level just distal to the left subclavian artery. SCI was continued for 12 min.

For inserting the microdialysis probe, the dura mater was torn. The tip of the microdialysis probe was placed in the ventral horn at an angle of 15° to the vertical line, 1.2 mm to the lateral side of the center of the spinal cord, and at a depth of 1.4 mm.

Abbreviations: SCI, spinal cord ischemia; MEP, motor-evoked potential.

Supplemental Methods

General anesthesia

In Experiment 1, 30 rats were administered 1.5 g/kg urethane (Sigma-Aldrich, Co. LLC., St. Louis, MO) intraperitoneally to induce anesthesia. In Experiment 2, 30 rats were initially anesthetized with 5.0% sevoflurane (NIKKO Pharmaceutical Co. Ltd., Gifu, Japan) until completion of tracheotomy, and anesthesia was maintained with 3.0% sevoflurane after tracheotomy using an anesthesia machine (Aestiva 5-7100; Datex-Ohmeda Inc., Madison, WI). In Experiment 3 and 4, 18 rats were administered 1.5 g/kg urethane intraperitoneally. Same anesthetic drugs were used in the same experiment (not different among the groups). In Experiment 2, rats were required to recover from general anesthesia for the assessment of motor function, therefore, sevoflurane was used for quick recovery from anesthesia.

Surgical preparation

After tracheotomy, the rats were ventilated with a tidal volume of 10 mL/kg (SN480-7; Shinano Co., Tokyo, Japan). The respiratory rate was adjusted to maintain the partial pressure of carbon dioxide between 35–40 mmHg. Rectal temperature was continuously monitored using a thermometer (DCBcIII; Technowood Co. Ltd., Tokyo, Japan) and

maintained around 37°C by using a heat lamp during the experiment.

Spinal Cord Ischemia

Essential information was depicted in the main manuscript. The duration of spinal cord ischemia (SCI) of 12 min was determined referring to the previous studies,^{1,2} as a longer duration of aortic clamping would be associated with a higher mortality rate during the experiment.

In Vivo Spinal Microdialysis

Essential information was depicted in the main manuscript. To measure the glutamate concentration in the ventral horn, the insertion angle and depth of the microdialysis probe were set on the basis of the atlas and confirmed before commencement of the experiment.

For measuring the glutamate concentration, 10 μ L of a 4-mM *o*-phthalaldehyde/2-mercaptoethanol derivative was added to 20 μ L of the dialysate, and the mixture was injected into an electrochemical detector (HTEC-500; Eicom). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0) and methanol (7:3 v/v) containing 5.0 mg/L ethylenediaminetetraacetic acid disodium salt. Glutamate was

separated on an analytical reverse-phase column (Eicompak SC-5ODS; 150 mm × 2.1 mm ID; Eicom), which was maintained at 30°C. A salt bridge Ag–AgCl reference electrode and a pure graphite working electrode (WE-PG; Eicom) were used for measurement of glutamate concentration. The working potential was set at 600 mV. The signal from the current-potential converter was filtered and integrated by the chromatography data software (PowerChrom; AD-Instruments Pty Ltd., Castle Hill, New South Wales, Australia).

Motor Evoked Potential

Essential information was depicted in the main manuscript. The stimulation and sensing leads were connected to an electrical stimulator (NS-101; UNIQUE MEDICAL). The strength and duration of the stimulus were 2.0–2.5 mA and 0.1 ms, respectively, set at train-of-five stimuli; the stimulus interval was 2 ms. The signals obtained were amplified 10,000–20,000 times and filtered between 5–300 Hz using a filter (EBA-100; UNIQUE MEDICAL).

Motor function

The grade of ambulation was defined as follows: 0, normal; 1, ataxic gait (the toes were

flat while walking); 2, knuckle-walk; 3, some movement in the hindlimb (unable to knuckle-walk); and 4, no movement in the hindlimb. The grade of placing/stepping reflex was defined as follows: 0, normal; 1, weak; and 2, no stepping. For each rat, a motor deficit index (MDI) score, the sum of ambulation and reflex scores, was calculated 48 h after reperfusion.

Histopathologic Evaluation

Essential information was depicted in the main manuscript. After physiologic evaluation, all rats were euthanized with an overdose of urethane (3 g/kg), following which the chest was opened. Ringer's solution (100 mL) and 3.7% formaldehyde (150 mL) were perfused from the descending thoracic aorta at a level just distal to the left subclavian artery. Following perfusion, the spinal cord was removed and preserved in formaldehyde for 72 h. After this period, the segment of 10th thoracic spinal cord was dissected, embedded in paraffin, and sectioned at a thickness of 3 μ m. The slides were stained with Nissl stain. For histopathologic evaluation, the numbers of normal and dark-stained motor neurons in the ventral horn were counted at a magnification of 20 \times .^{3,4} Normal motor neurons were defined as cells containing the Nissl substance in the cytoplasm, prominent nucleoli, and loose chromatin. In order to minimize observer

variability, the numbers of normal and dark-stained motor neurons were determined by calculating the average numbers from three different slides.² The histopathological slides were analyzed by a medical doctor (A.M.) who got trained of histopathologic evaluation, and the treatment group of each slide was masked by K.S; who marked newly generated randomized slide numbers on the correspondence table. The evaluation was performed by A.M. twice in each slide. Intra-observer analysis was performed by using intraclass correlation coefficient (ICC) and Bland-Altman analysis.⁵

Immunofluorescence

Essential information was depicted in the main manuscript. Immunofluorescence was performed to examine the association of N-methyl-D-aspartate receptor 2B (NR2B) subunit and adenosine A₁ receptor with the mechanisms of SCI and RIPC. Spinal cord extraction was performed by the same method in the *Histopathologic Evaluation* section. After deparaffinization, the sections were incubated in 0.3% Triton X-100 (Sigma-Aldrich) for 15 min, followed by 30% bovine serum albumin (Sigma-Aldrich) for 30 min. After that, the sections were incubated with primary antibody of rabbit polyclonal anti-NR2B (ab65783, 1:200; Abcam, Cambridge, UK) and anti-adenosine A₁ receptor (ab82477, 1:100; Abcam) for 60 min at room temperature. Then, the sections

were incubated with secondary antibody (Alexa Fluor 488, 1:200; Abcam) for 60 min at room temperature. The sections were examined by laser microscopes at a magnification of 4× and 20×.

Oxidative stress evaluation

Essential information was depicted in the main manuscript. The hydroperoxides are intermediate oxidative products of lipids, peptides and amino acids, and their amount is related to that of the free radicals.^{6,7} To measure the amount of hydroperoxides, the diacron-reactive oxygen metabolites (d-ROMs) test was performed using FREE carpe diem (Wismerll CO, Ltd., Tokyo, Japan), as previously described.⁶⁻⁸ Twenty μ L of blood and perfusate were dissolved in an acetate-buffered (pH 4.8) in order to generate newly formed radicals. Then, the amount of these radicals was measured through spectrophotometric procedures at 505 nm.

Supplemental Results

Essential information was depicted in the main manuscript.

A total of 123 rats were used in the experiment (Experiment 1:37, Experiment 2:49, Experiment 3:18, and Experiment 4:19). Among them, 27 rats died because of any of the followings: bleeding (8), apnea after anesthetics or MK-801 administration (4), airway problems (14), or unknown reason (1). Finally, 96 rats were analyzed (Experiment 1 and 2: 30 rats in each; 5 groups; n=6 in each group, Experiment 3 and 4: 18 rats in each; 3 groups; n=6 in each group).

Supplemental Discussion

Essential information was depicted in the main manuscript.

Glutamate and neuronal injury

Glutamate plays an important role in intermediary metabolism in every organ linking carbohydrate and amino acid metabolism by the tricarboxylic acid cycle.⁹ Previous studies showed the protective effect of glutamate on cardiac function, when added to cardioplegia and reperfusion solutions.^{10,11} This may be because glutamate enhances ATP generation by improving glycolysis and substrate-level phosphorylation.¹¹ In the central nervous system, glutamate also acts as a neurotransmitter. However, overaccumulation of glutamate after central nervous system insult leads to overstimulation of the NMDA receptor, followed by neuronal injury, called excitotoxicity.^{4,12,13} Excessive NMDA receptor activation can lead to Ca^{2+} homeostasis disorder, which induces the activation of several cytokines and proteases, thus causing free radical and nitric oxide (NO) production.^{14,15} Activation of these signal pathways is associated with neuronal cell death.

Oxidative stress

In the current study, RIPC attenuated the increase in glutamate concentration following SCI probably by preventing excessive NMDA receptor activation. The results of hydroperoxides measurement in the spinal cord support this hypothesis, as excessive NMDA receptor activation can lead to free radical production. In the current study, the amount of hydroperoxides of the spinal cord in the SCI group were significantly higher than that in the Sham group, while hydroperoxides in the spinal cord did not increase significantly in the RIPC/SCI group (Supplemental Figure 4). These results indicate that RIPC suppresses oxidative stress in the spinal cord after SCI, which might reflect the modulation of the NMDA receptor-mediated signaling pathways. On the contrary, there was no significant difference in the amount of blood hydroperoxides between the SCI and RIPC/SCI groups. The reason why the amount of blood hydroperoxides was not elevated may be the followings: in blood, systemic antioxidant systems, such as superoxide dismutase and vitamin C, are well-developed.¹⁶ Thus, the elevation of free radicals might be masked. On the other hand, in the spinal cord, the antioxidant system is not well-developed. Therefore, SCI induced the increased hydroperoxides in the spinal cord.

NMDA receptor antagonist

NMDA receptor antagonist itself is considered harmless to neuronal cells, and has a protective effect against ischemia-perfusion injury in the spinal cord.^{17,18} However, previous studies also showed that NMDA receptor antagonist interferes with the effect of NMDA-induced preconditioning, when administered before preconditioning.^{19,20} Considering the results of the current study, pre-administration of MK-801 canceled the protective effect of remote ischemic preconditioning. We assumed that NMDA receptor might be involved in the mechanism of remote ischemic preconditioning, which was supported by the result of immunofluorescence. In the current study, MK-801/SCI group was already included and confirmed that pre-administration of MK-801 (60min before RIPC, 140min before induction of SCI) itself has no effect on the ischemia-perfusion injury in the spinal cord. Therefore, another rat group with only NMDA receptor antagonist administration (without SCI) was not included in this study.

Comparison with the previous studies

Some previous studies have investigated the protective effect of RIPC on spinal cord ischemia-reperfusion injury. Haapanen, et al.²¹ investigated whether RIPC has the protective effect at 24 hours after spinal cord ischemia from the immunohistochemical

point of view. They showed that RIPC did not provide significant differences in the antioxidant markers and oxidative stress markers of the spinal cord, and serum reactive oxygen species. The same research group previously reported that RIPC exhibited protective effect against oxidative stress at 6 hours after spinal cord ischemia.²² Considering these results, the antioxidative effect of RIPC may not continue for more than 24 hours. However, the previous study²¹ did not assess motor function, and thus, it did not provide convincing conclusions about the impact of RIPC on the spinal cord ischemia. Previous study²³ as well as our study showed that RIPC prevented the impairment of motor function at 48 hours after spinal cord ischemia. Furthermore, in the current study, direct microdialysis in the ventral horn was performed, which could detect acute changes in glutamate concentration, and reveal the mechanism of RIPC in spinal cord protection as well as the detailed time course of spinal cord ischemia, as shown in our results.

RIPC in Clinical settings

In clinical settings, the protective effect of RIPC has been demonstrated in multiple organs.²⁴⁻²⁶ In contrast, some previous studies have contradicted the protective effect of RIPC, especially when propofol is used.^{27,28} Considering that previous studies²⁹ have

demonstrated the protective effect of RIPC on kidney injury without propofol, it is possible that propofol prevents the effect of RIPC against IRI.²⁷ Since propofol inhibits NMDA receptor activation,³⁰ it would interfere with the effect of RIPC in the same way as the NMDA receptor antagonist did in our study. Considering our results demonstrating that pre-administration of an NMDA receptor antagonist cancels the effect of RIPC, we might want to be cautious about using NMDA receptor antagonists when applying RIPC for surgical patients.

The results of the current study demonstrated the protective effect of RIPC on spinal cord ischemia-reperfusion injury after aortic cross-clamping and reperfusion. Thus, it may be beneficial for open thoracoabdominal aortic aneurysm repair. On the other hand, thoracic endovascular aortic repair (TEVAR) does not need aortic cross-clamping. The pathogenesis of SCI after TEVAR is multifactorial. Two theories are suggested behind the mechanism of SCI. First, remodeling of the collateral blood supply network is inadequate to maintain the perfusion in the spinal cord. Second, atheroembolism may be caused by aortic plaque material flushed into the segmental arteries.³¹ Delayed paraplegia may occur because of the embolism in type II endoleaks.³² Therefore the mechanisms of SCI between open surgery and TEVAR may be different. However, in both open surgery and TEVAR, decreased blood flow in the

spinal cord will result in glutamate accumulation, followed by excessive activation of NMDA receptor and spinal cord injury, called excitotoxicity. Our results indicate that RIPC prevents spinal cord ischemia-induced extracellular glutamate increase in the ventral horn, and also suppressed NR2B expression. Thus, we think that RIPC can be beneficial in both open surgery and TEVAR.

Supplemental References

1. Cavalcante LP, Ferreira SG, Pereira DR, Moraes SR, Simas R, Sannomiya P, et al. Acute administration of oestradiol or progesterone in a spinal cord ischaemia-reperfusion model in rats. *Interact Cardiovasc Thorac Surg*. 2018;26:196-201.
2. Kurita N, Kawaguchi M, Horiuchi T, Inoue S, Sakamoto T, Nakamura M, et al. An evaluation of white matter injury after spinal cord ischemia in rats: a comparison with gray matter injury. *Anesth Analg*. 2005;100:847-54.
3. Kakinohana M, Marsala M, Carter C, Davison JK, Yaksh TL. Neuraxial morphine may trigger transient motor dysfunction after a noninjurious interval of spinal cord ischemia: a clinical and experimental study. *Anesthesiology*. 2003;98:862-70.
4. Kakinohana M, Kakinohana O, Jun JH, Marsala M, Davison KJ, Sugahara K. The activation of spinal N-methyl-D-aspartate receptors may contribute to degeneration of spinal motor neurons induced by neuraxial morphine after a noninjurious interval of spinal cord ischemia. *Anesth Analg*. 2005;100:327-34.
5. Popovic ZB, Thomas JD. Assessing observer variability: a user's guide. *Cardiovasc Diagn Ther*. 2017;7:317-24.
6. Cornelli U, Terranova R, Luca S, Cornelli M, Alberti A. Bioavailability and antioxidant activity of some food supplements in men and women using the D-Roms test as a marker of oxidative stress. *J Nutr*. 2001;131:3208-11.
7. Tsuchiya M, Sato EF, Inoue M, Asada A. Open abdominal surgery increases intraoperative oxidative stress: can it be prevented? *Anesth Analg*. 2008;107:1946-52.
8. Uekusa H, Miyazaki C, Kondo K, Harada N, Nomoto J, Sugo N, et al. Hydroperoxide in internal jugular venous blood reflects occurrence of subarachnoid hemorrhage-induced delayed cerebral vasospasm. *J Stroke Cerebrovasc Dis*. 2014;23:2217-24.
9. Schousboe A, Scafidi S, Bak LK, Waagepetersen HS, McKenna MC. Glutamate metabolism in the brain focusing on astrocytes. *Adv Neurobiol*. 2014;11:13-30.
10. Pisarenko OI, Solomatina ES, Studneva IM, Ivanov VE, Kapelko VI, Smirnov VN. Protective effect of glutamic acid on cardiac function and metabolism during cardioplegia and reperfusion. *Basic Res Cardiol*. 1983;78:534-43.
11. Rosenkranz ER, Okamoto F, Buckberg GD, Vinten-Johansen J, Robertson JM, Bugyi H. Safety of prolonged aortic clamping with blood cardioplegia. II. Glutamate enrichment in energy-depleted hearts. *J Thorac Cardiovasc Surg*. 1984;88:402-10.
12. Redmond JM, Gillinov AM, Zehr KJ, Blue ME, Troncoso JC, Reitz BA, et al.

- Glutamate excitotoxicity: a mechanism of neurologic injury associated with hypothermic circulatory arrest. *J Thorac Cardiovasc Surg.* 1994;107:776-87.
13. Rokkas CK, Helfrich LR, Jr., Lobner DC, Choi DW, Kouchoukos NT. Dextrorphan inhibits the release of excitatory amino acids during spinal cord ischemia. *Ann Thorac Surg.* 1994;58:312-20.
 14. Arundine M, Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium.* 2003;34:325-37.
 15. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. *Cell Calcium.* 2010;47:122-9.
 16. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J.* 2012;5:9-19.
 17. Martinez-Arizala A, Rigamonti DD, Long JB, Krammer JM, Holaday JW. Effects of NMDA receptor antagonists following spinal ischemia in the rabbit. *Exp Neurol.* 1990;108:232-40.
 18. Gomez-Pinilla F, Tram H, Cotman CW, Nieto-Sampedro M. Neuroprotective effect of MK-801 and U-50488H after contusive spinal cord injury. *Exp Neurol.* 1989;104:118-24.
 19. Bond A, Lodge D, Hicks CA, Ward MA, O'Neill MJ. NMDA receptor antagonism, but not AMPA receptor antagonism attenuates induced ischaemic tolerance in the gerbil hippocampus. *Eur J Pharmacol.* 1999;380:91-9.
 20. Rejdak R, Rejdak K, Sieklucka-Dziuba M, Stelmasiak Z, Grieb P. Brain tolerance and preconditioning. *Pol J Pharmacol.* 2001;53:73-9.
 21. Haapanen HJ, Herajarvi J, Honkanen HP, Mustonen C, Tuominen H, Puistola U, et al. Immunohistochemical Analysis of the Spinal Cord Ischemia- Effect of Remote Ischemic Preconditioning in a Porcine Model. *Heart Surg Forum.* 2018;21:E209-e14.
 22. Herajarvi J, Anttila T, Sarja H, Mustonen C, Haapanen H, Makela T, et al. Exploring Spinal Cord Protection by Remote Ischemic Preconditioning: An Experimental Study. *Ann Thorac Surg.* 2017;103:804-11.
 23. Dong HL, Zhang Y, Su BX, Zhu ZH, Gu QH, Sang HF, et al. Limb remote ischemic preconditioning protects the spinal cord from ischemia-reperfusion injury: a newly identified nonneuronal but reactive oxygen species-dependent pathway. *Anesthesiology.* 2010;112:881-91.
 24. Meng R, Asmaro K, Meng L, Liu Y, Ma C, Xi C, et al. Upper limb ischemic preconditioning prevents recurrent stroke in intracranial arterial stenosis. *Neurology.* 2012;79:1853-61.
 25. Zimmerman RF, Ezeanuna PU, Kane JC, Cleland CD, Kempananjappa TJ, Lucas

- FL, et al. Ischemic preconditioning at a remote site prevents acute kidney injury in patients following cardiac surgery. *Kidney Int.* 2011;80:861-7.
26. Garcia-de-la-Asuncion J, Bruno L, Perez-Griera J, Galan G, Morcillo A, Wins R, et al. Remote Ischemic Preconditioning Decreases Oxidative Lung Damage After Pulmonary Lobectomy: A Single-Center Randomized, Double-Blind, Controlled Trial. *Anesth Analg.* 2017;125:499-506.
 27. Kottenberg E, Thielmann M, Bergmann L, Heine T, Jakob H, Heusch G, et al. Protection by remote ischemic preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol - a clinical trial. *Acta Anaesthesiol Scand.* 2012;56:30-8.
 28. Meybohm P, Bein B, Brosteanu O, Cremer J, Gruenewald M, Stoppe C, et al. A Multicenter Trial of Remote Ischemic Preconditioning for Heart Surgery. *N Engl J Med* 2015;373:1397-407.
 29. Zarbock A, Kellum JA, Van Aken H, Schmidt C, Kullmar M, Rosenberger P, et al. Long-term Effects of Remote Ischemic Preconditioning on Kidney Function in High-risk Cardiac Surgery Patients: Follow-up Results from the RenalRIP Trial. *Anesthesiology.* 2017;126:787-98.
 30. Kozinn J, Mao L, Arora A, Yang L, Fibuch EE, Wang JQ. Inhibition of glutamatergic activation of extracellular signal-regulated protein kinases in hippocampal neurons by the intravenous anesthetic propofol. *Anesthesiology.* 2006;105:1182-91.
 31. Awad H, Ramadan ME, El Sayed HF, Tolpin DA, Tili E, Collard CD. Spinal cord injury after thoracic endovascular aortic aneurysm repair. *Can J Anaesth* 2017;64:1218-35.
 32. Etz CD, Weigang E, Hartert M, Lonn L, Mestres CA, Di Bartolomeo R, et al. Contemporary spinal cord protection during thoracic and thoracoabdominal aortic surgery and endovascular aortic repair: a position paper of the vascular domain of the European Association for Cardio-Thoracic Surgery. *Eur J Cardiothorac Surg.* 2015;47:943-57.