Anesthesiology 2010; 112:881–91

Limb Remote Ischemic Preconditioning Protects the Spinal Cord from Ischemia–Reperfusion Injury

A Newly Identified Nonneuronal but Reactive Oxygen Species–dependent Pathway

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ABSTRACT

Background: It remains to be established whether spinal cord ischemic tolerance can be induced by limb remote ischemic preconditioning (RIPC), and the mechanisms underlying the neuroprotective effects of RIPC on the spinal cord need to be clarified.

Methods: Spinal cord ischemia was studied in New Zealand White rabbits. In experiment 1, all rabbits were subjected to 20-min spinal cord ischemia by aortic occlusion. Thirty minutes before ischemia, rabbits were subjected to sham intervention or RIPC achieved by bilateral femoral artery occlusion (10 min ischemia/10 min reperfusion, two cycles). Dimethylthiourea (500 mg/kg, intravenously), a hydroxyl radical scavenger, or vehicle was given 1 h before RIPC. Antioxidant enzyme activity was measured along with spinal cord histology and neurologic function. In experiment 2, rabbits were subjected to spinal cord ischemia, with or without RIPC. In addition, rabbits were pretreated with various doses of hexamethonium.

Results: RIPC improved neurologic function and reduced histologic damage. This was associated with increased endogenous antioxidant activity. Dimethylthiourea inhibited the protective effects of RIPC. In contrast, there was no effect of hexamethonium on the protective effect of RIPC.

Conclusions: An initial oxidative stress acts as a trigger to upregulate antioxidant enzyme activity, rather than the neural pathway, and plays an important role in the formation of the tolerance against spinal cord ischemia by limb RIPC.

What We Already Know about This Topic

- Temporary ischemia of one organ may protect ischemic injury to another
- This protective effect may be due to humoral or neural factors

What This Article Tells Us That Is New

- In rabbits, spinal cord injury from ischemia was reduced by previous temporary lower limb ischemia
- This protection was abolished by an inhibitor of oxidative stress but not by a ganglionic blocker, indicating the importance of humoral factors

OPERATIONS on the thoracoabdominal aorta and spine may result in spinal cord ischemia–reperfusion injury leading to paraplegia.[1,2] Although a number of studies have shown that the risk of postoperative paraplegia was decreased with the application of new techniques, preventing spinal cord ischemic injury is still a big concern in the thoracic aorta surgery.[3]

In the past decades, ischemic preconditioning (IPC) has been reported to minimize ischemia–reperfusion injury in many organs, including the spinal cord in animal models.[4,5] Unfortunately, the idea of exposure to brief periods of ischemia before an anticipated ischemic event in a vital organ, such as cerebral and spinal cord, is not a viable option in clinical practice. Recent studies have found that IPC-induced ischemic tolerance occurred not only within the same piece of tissue but also between different regions and different organs. An ischemia performed in one organ may also protect against a subsequent lethal ischemia in another distant organ. This phenomenon is referred to as remote IPC (RIPC).[6] Because RIPC is a more amenable and less invasive approach than classic IPC, it was first applied to coronary arterial surgery and received satisfactory results.[7,8]

In our previous study, we demonstrated that limb RIPC can induce ischemic tolerance against cerebral and spinal cord ischemia–reperfusion injury in rats and rabbits.[9,10] However, the mechanism of ischemic tolerance on spinal cord ischemia induced by limb RIPC is still unclear. Both humoral factors and neural pathways have been proposed to

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Received from the Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China. Submitted for publication April 15, 2009. Accepted for publication December 2, 2009. Supported by Grant 30725039 from the National Science Foundation for Distinguished Young Scholars (Beijing, China) (to Dr. Xiong) and Grant 30300329 from the National Natural Science Foundation of China (Beijing, China) (to Dr. Dong).

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be involved in the fundamental mechanisms underlying RIPC cardiac protection.[11] Opioids, oxygen-free radicals, and endocannabinoid are reported to be humoral mediators of RIPC in cardiac ischemia protection. Among these humoral factors, oxygen-free radicals seem to play an important role in the induction of ischemic tolerance by RIPC.[12,13] Gho *et al.*[14] also found that the reduction in myocardial infarct size induced by RIPC could be reversed in the presence of the ganglion blocker, hexamethonium (HEX). This indicated that a neural pathway might be involved in the mechanism of inducing ischemic tolerance by RIPC. However, the results of subsequent studies are controversial.[15] It seems that the reverse of RIPC cardiac protection by HEX depends on when the drug was given. Whether the humoral pathway or the neural pathway plays a crucial role in the RIPC protective effect on spinal cord ischemia is unclear. Furthermore, it remains to be established whether the free radicals generated by the brief ischemia of the limb play a crucial role in inducing protection against spinal cord ischemia.

In this study, we have tested the hypothesis that oxidative stress is a crucial mediator of limb RIPC neuroprotection on spinal cord ischemia by using a free-radical scavenger, dimethylthiourea (DMTU), in a rabbit model. Meanwhile, the role of neuronal pathway in the protective effect of RIPC on spinal cord ischemia is also evaluated by administration of HEX before RIPC.

Materials and Methods

Animals and Surgical Preparation

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of our institutes. The animals were studied at Xijing Hospital of the Fourth Military Medical University (Xi'an, China).

One hundred nineteen male New Zealand White rabbits (weight, 2.1–2.3 kg) were used in this study. After an overnight fast with unrestricted access to water, the rabbits were anesthetized with 2.0% isoflurane in an oxygen–room air mixture. After induction, rabbits were maintained with 1.5% isoflurane delivered by mask while spontaneously breathing. An ear vein catheter was inserted, and lactated Ringer's solution ($4\ ml \cdot kg^{-1} \cdot h^{-1}$) was administered intravenously. Another catheter was inserted into the contralateral ear vein for blood sampling. In all animals, a 24-gauge catheter was inserted into the ear artery to measure proximal blood pressure and sample blood gases. Another catheter was inserted into the left femoral artery to measure distal blood pressure. After all cannulae were placed, 400 units of heparin was injected intravenously. Blood pressure was monitored continuously using a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Medical, Inc., Redmond, WA). Heart rate was calculated from the blood pressure waveform displayed on the monitor. According to previous reports, the normal rabbit body temperature ranges between 38.3° and 39.4°C.[16] Therefore, rectal temperature was maintained at 38.5° ± 0.5°C using a heating blanket and overhead lamp. Arterial blood was sampled for determination of arterial oxygen tension (Pa_{O_2}), arterial carbon dioxide tension (Pa_{CO_2}), *p*H, and plasma glucose. Arterial blood gases were measured using the OMNI Modular System (AVL List GmbH Medizintechnik, Kleiststraβe, Graz, Austria).

Spinal Cord Ischemia

The induction of spinal cord ischemia was performed as previously described by our group.[12,17] In brief, the abdominal aorta was exposed at the level of the left renal artery through a 3- to 4-cm medial incision. Small-diameter plastic tubing was placed around the aorta just distal to the left renal artery. The ends of the tubing were threaded through a small plastic button and then through a plastic tube of large diameter, forming a snare ligature. Aortic occlusion was performed by pulling and clamping the small tube around the aorta. After the occlusion, distal blood pressure decreased immediately, and the pulsatility disappeared. Ischemia lasted 20 min. At the end of the ischemic period, the tubing was released to restore the flow through the aorta. The abdominal wall and limb incisions were closed with sutures. The local infiltration around the wound with 0.25% bupivacaine hydrochloride was applied for postoperative analgesia. Isoflurane was discontinued. The infusion of lactated Ringer's solution was continued until the animals began to drink. Immediately after the operation, an antibiotic (40,000 IU gentamicin) was administered intramuscularly. The animals were then returned to their home cages and observed for 2 days. Bladder contents were expressed manually as required.

Experimental Protocol

This study consisted of two experiments. Experiment 1 was designed to determine whether the protective effect of limb RIPC against spinal cord ischemia is mediated by upregulating antioxidant enzyme activities. Experiment 2 was undertaken to elucidate the effect of ganglion blocker HEX on the ischemic tolerance against spinal cord ischemia induced by limb RIPC.

Experiment 1. Effect of DMTU on the Induction of Ischemic Tolerance by Limb RIPC and the Antioxidant Enzyme Activities after RIPC

Part I. The design of experiment 1 is shown in figure 1A. A total of 24 male New Zealand White rabbits were randomly assigned to four groups using random numbers (n = 6 in each). The animals in the RIPC/no DMTU and RIPC + DMTU groups received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 minutes before spinal cord ischemia. The modified round cuff devices (2-cm wide) were used bilaterally to occlude the femoral arteries in this study. The RIPC was induced by inflating the cuff to 200 mmHg. The animals in the no RIPC/no

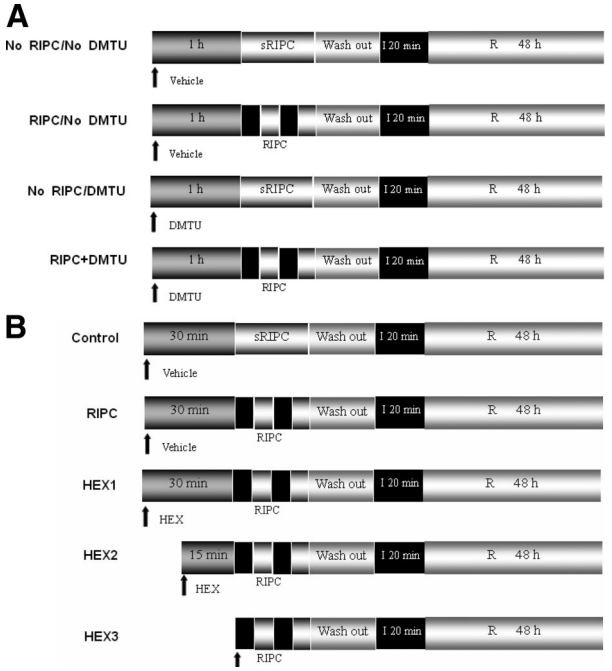

Fig. 1. The experiment protocols (*A*, experiment 1; *B*, experiment 2). RIPC = remote ischemic preconditioning; sRIPC = sham remote ischemic preconditioning; DMTU = dimethylthiourea; HEX = hexamethonium; I = spinal cord ischemia; R = reperfusion.

DMTU and no RIPC/DMTU groups only underwent spinal cord ischemia without pretreatment. DMTU (500 mg/kg) was intravenously administered to the animals in no RIPC/DMTU and RIPC + DMTU groups 1 h before pretreatment. The dose of DMTU was referred from previous reports.[12] All animals were subjected to spinal cord ischemia for 20 min. At 4, 6, 12, 24, and 48 h after reperfusion, the hind limb motor function of the rabbit was assessed. The histopathologic evaluation was performed 48 h after reperfusion. Superoxide dismutase (SOD) and catalase activities and malondialdehyde content in serum were measured in the same animals. At the setting time points, 2 ml of venous blood was aspirated out of the ear vein. The blood loss was substituted with 2 ml of 0.9% sodium chloride solution.

Part II. For the SOD and catalase activities and malondialdehyde content measurement in spinal cord, a total of 65 male New Zealand White rabbits were randomly divided into four groups using random numbers: no RIPC/no DMTU, RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups. Five rabbits were killed at untreated condition as preischemic control. To minimize animal usage, four groups share this preischemic value as baseline. The others (n = 15 for each group) were subjected to the same procedures as shown in figure 1A. All animals were subjected to spinal cord ischemia for 20 min. The animals were killed by a lethal dose of pentobarbital (200 mg/kg) at 20 min after RIPC (or corresponding time point) and 6 and 24 h after reperfusion (n = 5 for each time point in each group). The time points of sample collection were determined according to our preliminary study and published data.[12] The spinal cord was removed less than 1 min after killing. Spinal cord tissues of L5–7 level were sampled and frozen at −70°C in a freezer for the determination of antioxidant enzyme activities and malondialdehyde content.

Experiment 2: Effect of HEX on the Induction of Ischemic Tolerance in Spinal Cord by Limb RIPC

The design of experiment 2 is shown in figure 1B. A total of 30 male New Zealand White rabbits were randomly assigned to five groups by using random numbers (n = 6 each): the control, RIPC, HEX1, HEX2, or HEX3 group. Rabbits in the RIPC, HEX1, HEX2, and HEX3 groups received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 min before spinal cord ischemia. HEX (a ganglion blocker, 20 mg/kg) was intravenously administered to the rabbits at 30, 15, and 0 min before the onset of RIPC in the HEX1, HEX2, and HEX3 groups, respectively. The animals in the control group received 20 min spinal cord ischemia but were not subjected to RIPC or drug treatment.

Neurologic and Histopathologic Evaluation

At 4, 6, 12, 24, and 48 h after reperfusion, the rabbits were neurologically assessed by an observer who was unaware of the grouping, using the modified Tarlov criteria[17]: 0 = no voluntary hind limb function; 1 = movement of joints perceptible; 2 = active movement but unable to stand; 3 = able to stand but unable to walk; and 4 = complete normal hind limb motor function.

After completion of the evaluation of hind limb motor function at 48 h after reperfusion, the animals were reanesthetized. Transcardiac perfusion and fixation were performed with 1,000 ml heparinized saline followed by 500 ml 10% buffered formalin. The lumbar spinal cord was removed and refrigerated in 10% phosphate-buffered formalin for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. Coronal sections of the spinal cord (L5 level) were cut at a thickness of 6 μm and stained with hematoxylin and eosin. Neuronal injury was evaluated at a magnification of 400× by an observer who was unaware of the grouping. Ischemic neurons were identified by cytoplasmic eosinophils with loss of Nissl substance and by the presence of pyknotic homogenous nuclei. In each slice, normal neurons in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) were counted in two sections for each animal and then averaged by an observer who was blinded to group assignment.

Measurement of Antioxidant Enzyme Activity

Blood samples were inverted gently several times and allowed to clot for 30 min at room temperature. Blood samples were centrifuged at 1,500*g* for 15 min to obtain serum. Serum samples were then stored at −20°C before analysis. Spinal cord tissues of L5–7 level were homogenized in cold saline with a weight-to-volume ratio of 1:10. The measurements of

both antioxidant enzyme activities and malondialdehyde content in tissue homogenates and serum samples were performed according to the technical manual of the detection kits (Jianchen Biologic Institute, Nanjing, Jiangsu, China).[12] SOD activity was measured after the reduction of nitrite by a xanthine–xanthine oxidase system, which is a superoxide generator. In brief, spinal cord homogenate or serum was diluted 1:400 with 10 mM phosphate buffer, pH 7.00. Approximately 25 μl of dilution was mixed with 850 μl of substrate solution containing 0.05 mM xanthine sodium and 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride in a buffer solution containing 50 mM 3-cyclohexylamino-1-propanesulfonic acid and 0.94 mM EDTA, pH 10.2. Then, 125 μl of xanthine oxidase (80 U/l) was added to the mixture, and absorbance increase was followed at 505 nm for 3 min against air. Approximately 25 μl of phosphate buffer or 25 μl of various standard concentrations in place of sample was used as blank or standard determinations. One unit of SOD is defined as the amount that shows 50% inhibition. The activity was expressed as units per milliliter for serum and units per milligram of protein for tissue homogenate. Serum catalase activity was measured polarographically as the rate of production of oxygen from H_2O_2. One unit of catalase activity was defined as the amount of catalase that consumed 1 pmol H_2O_2/min at 25°C, pH 7.0. Catalase activity was assayed by measuring absorbance at 240 nm using an ultraviolet light spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and was expressed as units per milliliter for serum and units per gram of protein for tissue homogenate. The definition of its activity was based on the hydrogen peroxide decomposition rate at 240 nm in the reactive mixture, of which the absorbance was between 0.5 and 0.55.

Determination of Malondialdehyde Levels

Malondialdehyde was determined by the method of Chen *et al.*[18] The assay mixture consisted of 0.1 ml serum or tissue homogenate, 0.4 ml 0.9% NaCl, 0.5 ml 3% sodium dodecyl sulfate, 3 ml thiobarbituric acid reagent (containing equal parts of 0.8% aqueous thiobarbituric acid and acetic acid) and was heated for 75 min at 95°C. Thereafter, the mixture was added to 1 ml of cold 0.9% NaCl and cooled in tap water and extracted by adding 5 ml of *n*-butanol. After centrifugation at 3,000*g* for 15 min, the butanol phase was assayed spectrophotometrically at 532 nm. Amounts of 0, 20, 40, 60, and 80 nmol of tetramethoxypropane served as the external standard and were assayed in the previously described fashion. Malondialdehyde content was expressed as micromolar per liter for serum and millimolar per milligram of protein for tissue homogenate.

Statistical Analysis

Blood pressure, blood gases, plasma glucose, antioxidant enzyme activities, and malondialdehyde content were compared by using a two-way analysis of variance, followed by a Student–Newman–Keuls test for multiple comparisons. The scores of hind limb motor function and the number of normal neurons in the anterior spinal cord were analyzed using a nonparametric method (Kruskal-Wallis test), followed by the Mann–Whitney U test with Bonferroni correction. The correlation of hind limb motor function scores and the number of normal neurons in the anterior spinal cord was analyzed using Spearman rank correlation. Statistical analysis of the data was performed using commercially available software program SPSS version 13.0 (SPSS Inc., Chicago, IL). All the testings were two tailed. A P value less than 0.05 was considered statistically significant.

Results

Experiment 1

Physiologic Variables. Physiologic variables are shown in table 1. The hemodynamics, rectal temperature, arterial *p*H, Pa_{O_2}, Pa_{CO_2}, and plasma glucose were similar in all groups, regardless of the treatment patterns. The mean distal blood pressure was decreased to 8–10 mmHg after blocking the abdominal aorta. Ten minutes after the beginning of reperfusion, the value of the distal blood pressure was recovered to nearly preischemic level. During the experiments, the rectal temperature was maintained between 38° and 39°C by an overhead lamp.

Neurologic Outcome. The data of neurologic and histopathologic outcomes come from the animals in experiment 1, part 1. All animals survived until the final neurologic assessment at 48 h after reperfusion. Animals in the RIPC/no DMTU group showed higher hind limb motor function scores than those in the no RIPC/no DMTU group (table 2). There were no significant differences in hind limb motor function scores of animals in the no RIPC/no DMTU group in comparison with the no RIPC/DMTU and RIPC + DMTU groups ($P = 0.818$ and 0.699 for each comparison; table 2).

Histopathologic and Correlation of Neurologic and Histopathologic Outcomes. The normal neurons in the anterior spinal cord in the RIPC/no DMTU group (median = 54.7[41–60]) were more than those in the no RIPC/no DMTU group ($P = 0.002$). There was no difference in the number of normal neurons in the anterior spinal cord among the no RIPC/no DMTU (median = 9.1[4–15]), no RIPC/DMTU (median = 9[5–36]), and RIPC + DMTU (median = 5.5[3–16]) groups (fig. 2). There was a significant correlation between the final neurologic status at 48 h after reperfusion and the number of normal neurons in the anterior spinal cord ($r = 0.867$, $P = 0.001$).

Antioxidant Enzyme Activities and Malondialdehyde Level Changes in Serum after Limb RIPC. The activities of catalase and SOD in serum were at the same level among different groups at the basal condition. At 20 min after the RIPC, activities of catalase and SOD in serum in RIPC/no DMTU group were higher than that in the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups. Higher activities of catalase and SOD were found in the RIPC/no DMTU group compared with that in the other three groups at 20 min of ischemia

Table 1. Physiologic Variables during Preischemic and Postischemic State (n = 6)

	PAP, mmHg	DAP, mmHg	HR, beats/min	T, °C	Pa_{O_2}, mmHg	Pa_{CO_2}, mmHg	pH	G_s, mM
Preischemia								
No RIPC/no DMTU	76 ± 5	78 ± 6	255 ± 5	38.3 ± 0.4	226 ± 46	38 ± 4	7.38 ± 0.02	6.5 ± 0.8
RIPC/no DMTU	78 ± 6	74 ± 7	252 ± 12	38.4 ± 0.3	215 ± 50	37 ± 4	7.39 ± 0.05	6.4 ± 0.6
No RIPC/DMTU	84 ± 4	81 ± 5	259 ± 7	38.5 ± 0.5	225 ± 40	39 ± 5	7.40 ± 0.08	7.3 ± 1.1
RIPC + DMTU	76 ± 6	73 ± 5	258 ± 14	38.6 ± 0.2	230 ± 69	38 ± 7	7.35 ± 0.02	6.8 ± 0.7
Ischemia 10 min								
No RIPC/no DMTU	75 ± 4	9 ± 1	264 ± 16	38.5 ± 0.3	219 ± 37	41 ± 5	7.35 ± 0.08	6.6 ± 0.3
RIPC/no DMTU	81 ± 4	10 ± 0	263 ± 18	38.8 ± 0.4	225 ± 45	38 ± 5	7.39 ± 0.05	6.7 ± 0.9
No RIPC/DMTU	90 ± 6	10 ± 0	260 ± 20	38.2 ± 0.6	218 ± 37	40 ± 5	7.36 ± 0.04	6.7 ± 0.7
RIPC + DMTU	82 ± 6	9 ± 1	265 ± 18	38.6 ± 0.7	220 ± 49	39 ± 4	7.39 ± 0.03	6.9 ± 0.8
Reperfusion 10 min								
No RIPC/no DMTU	75 ± 5	70 ± 4	268 ± 14	38.4 ± 0.4	217 ± 44	38 ± 4	7.40 ± 0.05	7.0 ± 0.6
RIPC/no DMTU	72 ± 5	74 ± 5	261 ± 16	38.2 ± 0.5	208 ± 42	37 ± 3	7.41 ± 0.07	6.3 ± 0.1
No RIPC/DMTU	73 ± 5	71 ± 5	260 ± 12	38.6 ± 0.6	217 ± 55	38 ± 4	7.40 ± 0.06	6.6 ± 0.4
RIPC + DMTU	74 ± 5	75 ± 4	262 ± 18	38.4 ± 0.7	225 ± 37	37 ± 5	7.36 ± 0.05	6.8 ± 0.3

Data are presented as mean ± SD.

DAP = distal blood pressure; DMTU = dimethylthiourea; G_s = serum glucose concentration; HR = heart rate; MAP = mean arterial pressure; No RIPC/no DMTU = animal that received sham pretreatment 20 min before spinal cord ischemia; RIPC/no DMTU = animal that received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10 min/10-min interval 30 min before spinal cord ischemia; No RIPC/DMTU = animal that received sham pretreatment and dimethylthiourea preadministration (1 h before sham pretreatment) 20 min before spinal cord ischemia; Pa_{CO_2} = arterial carbon dioxide tension; Pa_{O_2} = arterial oxygen tension; PAP = proximal blood pressure; RIPC = remote ischemic preconditioning; RIPC + DMTU = animal that received RIPC and DMTU preadministration (1 h before sham pretreatment) 20 min before spinal cord ischemia; T = rectal temperature.

and 20 min and 6 h after reperfusion. No significant difference was observed among the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups at corresponding time points. In addition, SOD activities in all the three groups decreased at 20 min of ischemia and 20 min after reperfusion compared with preischemic state (fig. 3A). Catalase activities in the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups decreased at 20 min of ischemia compared with preischemic state (fig. 3B).

Malondialdehyde content in the serum in the RIPC/no DMTU group was slightly increased at 20 min after RIPC. After spinal cord ischemia, malondialdehyde content in the serum was significantly increased in the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups at 20 min and 6 h after reperfusion. However, the serum malondialdehyde content in the RIPC/no DMTU group was not significantly increased after spinal cord ischemia (fig. 3C). The serum malondialdehyde content in the RIPC/no DMTU group was significantly lower than that in other three groups at 6 h after reperfusion.

Table 2. Neurological Function Scores after Spinal Cord Ischemia

	6 h	24 h	48 h
No RIPC/no DMTU	1 (0–2)	0 (0–1)	0 (0–1)
RIPC/no DMTU	3 (3–4)*	4 (3–4)*	4 (3–4)*
No RIPC/DMTU	1 (1–2)	1 (0–2)	0 (0–2)
RIPC + DMTU	1 (0–2)	0 (0–1)	1 (0–1)

Data are presented as median (range).

* $P = 0.001$ *versus* no remote ischemic preconditioning (RIPC)/no dimethylthiourea (DMTU).

Antioxidant Enzyme Activities and Malondialdehyde Level Changes in Spinal Cord after Limb RIPC. A higher activity of catalase and SOD was found in the spinal cord of the RIPC/no DMTU group compared with the other three groups at 20 min after RIPC and 6 h after spinal cord ischemia–reperfusion. No significant difference was observed among the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups at corresponding time points. Catalase and SOD activities in the spinal cord of the RIPC/no DMTU group were decreased at 24 h after reperfusion. There was no significant difference in catalase and SOD activities in the spinal cord of the four groups at 24 h after reperfusion (figs. 4A and B).

Malondialdehyde content in the spinal cord was significantly increased at 6 and 24 h after reperfusion in all the four groups. However, the spinal cord malondialdehyde content in the RIPC/no DMTU group was significantly lower than that in the other three groups at 6 h after reperfusion. There was no significant difference in malondialdehyde content in the spinal cord of the four groups at 24 h after reperfusion (fig. 4C).

Experiment 2

Physiologic Variables and Neurologic Outcome. A marked decrease in arterial pressure and an increase in heart rate were

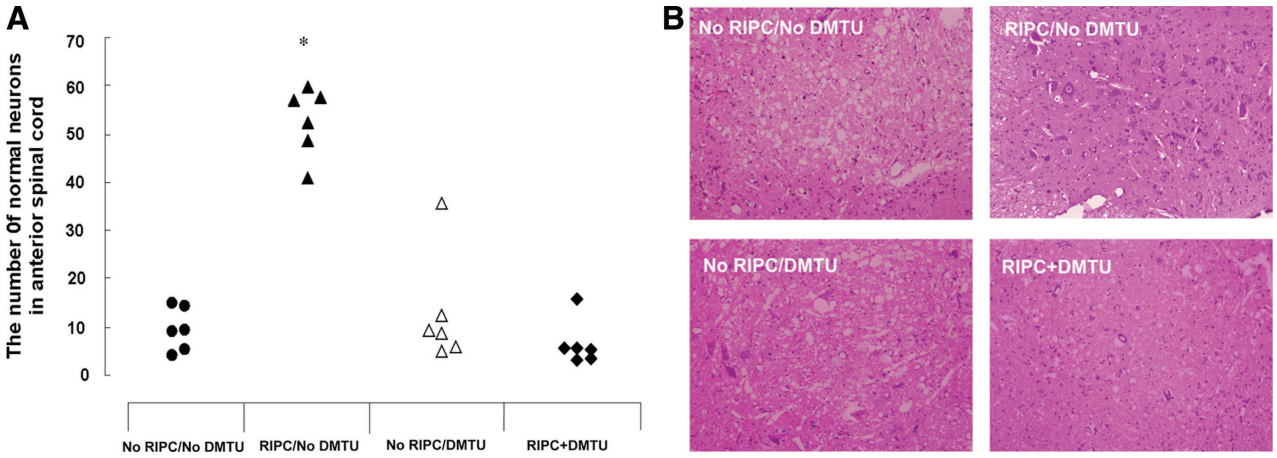

Fig. 2. (*A*) Experiment 1: Histopathologic outcome in each animal of the four groups at 48 h after reperfusion. The symbols *filled circle*, *filled triangle*, *open triangle*, and *filled diamond* represent animals in the no remote ischemic preconditioning (RIPC)/no dimethylthiourea (DMTU), RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups, respectively. * $P = 0.002$ *versus* no RIPC/no DMTU. (*B*) Representative photomicrographs of lumbar spinal cord sections (L5) at 48 h after reperfusion. Sections from a rabbit rated as Tarlov score 0 in the no RIPC/no DMTU group showed severe neuronal damage, as evidenced by disappearance of most of normal motor neurons in anterior spinal cord and extensive vacuolation of gray matter. Ischemic neurons were identified by cytoplasmic eosinophils with loss of Nissl substance and by the presence of pyknotic homogenous nuclei. Section from a rabbit rated as Tarlov score 4 in the RIPC/no DMTU group showed numerous normal motor neurons in the same area. The sections from the rabbits rated as Tarlov score 0–1 in the no RIPC/DMTU and RIPC + DMTU groups also showed severe neuronal damage in the anterior spinal cord and extensive vacuolation of gray matter. (Hematoxylin and eosin staining, magnification 200×). No RIPC/no DMTU = animal that received sham pretreatment before 20 minutes spinal cord ischemia; RIPC/No DMTU = animal that received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 min before spinal cord ischemia; No RIPC/DMTU = animal that received sham pretreatment and dimethylthiourea administration (1 hour before sham pretreatment) before 20 minutes spinal cord ischemia; RIPC + DMTU = animal that received RIPC and dimethylthiourea administration (1 hour before pretreatment) before 20 minutes spinal cord ischemia.

observed with the administration of HEX. This response peaked during the first 5 min and diminished by the 60-min duration. The arterial blood pressure and heart rate returned to baseline before spinal cord ischemia was induced. The hemodynamics, rectal temperature, arterial *p*H, Pa_{O_2}, and Pa_{CO_2} were similar in the setting time points in all groups, regardless of the treatment pattern (table 3).

All animals survived until the final neurologic assessment at 48 h after reperfusion. Animals in the RIPC group showed higher hind limb motor function scores than those in the control group. There were no significant differences in hind limb motor function scores of animals in the RIPC group in comparison with the HEX1, HEX2, and HEX3 groups (table 4).

Histopathologic and Correlation of Neurologic and Histopathologic Outcome. The normal neurons in the anterior spinal cord of the RIPC group (median = 54.8 [44–61]) were more than those in the control group (median = 9 [5–15]). However, no difference was found in the number of normal neurons in the anterior spinal cord among the RIPC, HEX1 (median = 55.8 [43–63]), HEX2 (median = 48.4 [35–59]), and HEX3 (median = 54.2 [32–60]) groups (fig. 5). There was a significant correlation between the final neurologic status at 48 h after reperfusion and the number of normal neurons in the anterior spinal cord ($r = 0.864$; $P = 0.001$).

Discussion

In this study, we found that spinal cord ischemic tolerance induced by limb RIPC was attenuated by administration of a free-radical scavenger, DMTU, before RIPC. The antioxidant enzyme activity measurement showed that RIPC induced an increase in the activity of major antioxidant enzymes (catalase and SOD) in the serum. The increase in catalase and SOD activities is accompanied by a transient increase of malondialdehyde levels in serum. DMTU pretreatment completely abolished the increase in catalase and SOD activity induced by RIPC. This indicates that the increase in antioxidant enzyme activities is closely related to the reactive oxygen species generated by RIPC. Although antioxidant enzyme activity decreased after spinal cord ischemia, significantly higher activities of catalase and SOD were seen in the RIPC/no DMTU group compared with the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups at corresponding time points. However, pretreatment with a ganglion blocker, HEX, did not reverse the protective effects of RIPC on spinal cord ischemic injury. Our results indicate that the involvement of enhanced endogenous antioxidant enzyme activities, especially SOD and catalase, in the induction of ischemic tolerance against spinal cord ischemia by RIPC involves a reactive oxygen species-mediated pathway. In contrast, a neural pathway does not seem to be involved in the formation of spinal cord ischemic tolerance induced by limb RIPC.

Spinal cord injury after a successful surgical operation on the thoracic aorta is an unpredictable but disastrous complication in human beings. The causes of acute spinal cord dysfunction are believed to be the result of spinal cord ischemia from hypoperfusion during aortic cross-clamping. Ex-

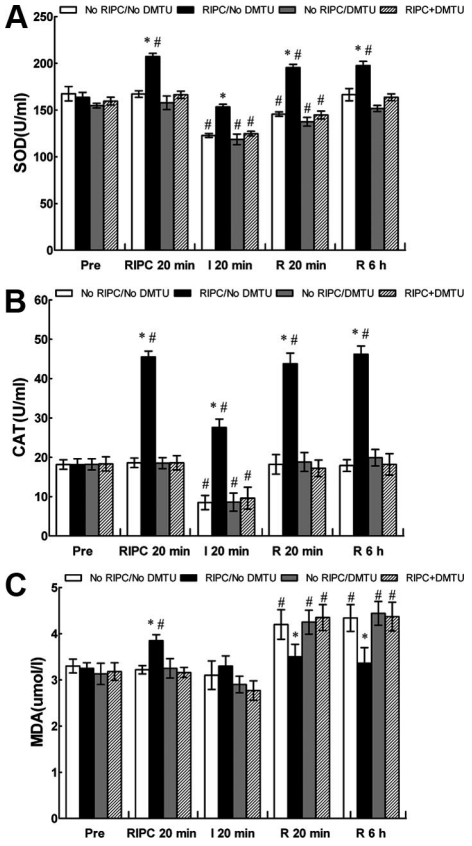

Fig. 3. Activities of superoxide dismutase, catalase, and content of malondialdehyde in the serum. (*A*) A higher activity of SOD was found in the RIPC/no DMTU group compared with that in the other three groups at 20 min after the RIPC ($P = 0.001$), 20 min of ischemia ($P = 0.001$), and 20 min ($P = 0.001$) and 6 h after reperfusion ($P = 0.001$). SOD activities in the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups decreased at 20 min of ischemia ($P = 0.001$) and 20 min after reperfusion ($P = 0.001$) compared with preischemic state. (*B*) A higher activity of catalase was found in the RIPC/no DMTU group compared with that in the other three groups at 20 min after the RIPC ($P = 0.001$), 20 min of ischemia ($P = 0.001$), and 20 min ($P = 0.001$) and 6 h after reperfusion($P = 0.001$). Catalase activities in the no RIPC/no DMTU ($P = 0.012$), no RIPC/DMTU ($P = 0.011$), and RIPC + DMTU ($P = 0.012$) groups decreased at 20 min of ischemia compared with preischemic state. (*C*) Malondialdehyde content in the serum in the RIPC/no DMTU group was increased at 20 min after RIPC ($P = 0.024$ *vs.* preischemic level). Malondialdehyde content in the serum was significantly increased in the no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU groups at 20 min and 6 h after reperfusion ($P = 0.001$ *vs.* preischemic level). The serum malondialdehyde content in the RIPC/no DMTU group was significantly lower than that in the other three groups at 6 h after reperfusion ($P = 0.013$, 0.011, and 0.014 *vs.* no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU, respectively). Data are presented as mean ± SD. * $P < 0.001$ *versus* No RIPC/No DMTU, # $P < 0.05$ *versus* preischemic state. No RIPC/no DMTU = animal that received sham pretreatment before 20 min spinal cord ischemia; RIPC/no DMTU = animal that received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 min before spinal cord ischemia; No RIPC/DMTU = animal that received sham pretreatment and dimethylthiourea administration (1 h before sham pretreatment) before 20 min spinal cord ischemia; RIPC + DMTU = animal that received RIPC and dimethylthiourea administration (1 h before pretreatment) before 20 min spinal cord ischemia. CAT = catalase; DMTU = dimethylthiourea; MDA = malondialdehyde; RIPC = remote ischemic preconditioning; SOD = superoxide dismutase; Pre = Pre RIPC; RIPC 20 min = 20 min after RIPC; I 20 min = 20 min of ischemia; R 20 min = 20 min after reperfusion; R 6 h = 6 h after reperfusion.

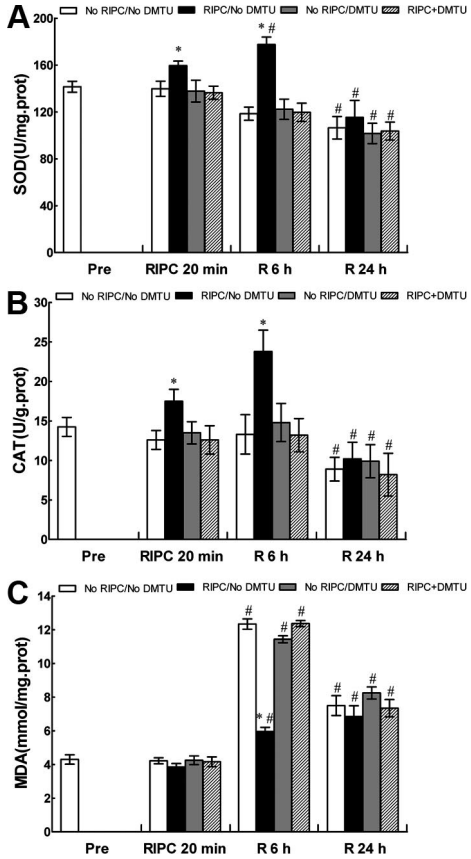

Fig. 4. Activities of superoxide dismutase (*A*), catalase (*B*), and content of malondialdehyde (*C*) in the spinal cord. (*A*) A higher activity of superoxide dismutase (SOD) was found in the spinal cord in the RIPC/no DMTU group compared with that in the other three groups at 20 min after RIPC ($P =$ 0.034, 0.026, and 0.021 *vs.* no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU; respectively) and 6 h after spinal cord ischemia–reperfusion ($P = 0.001$ vs. no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU). SOD activities in the spinal cord in the RIPC/no DMTU group were decreased at 24 h after reperfusion ($P = 0.002$ *vs.* preischemic level). (*B*) A higher activity of catalase was found in the spinal cord in the RIPC/no DMTU group compared with that in the other three groups at 20 min after RIPC ($P = 0.019$, 0.021, and 0.031 *vs.* no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU; respectively) and 6 h after spinal cord ischemia–reperfusion ($P = 0.001$ *vs.* no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU). Catalase activities in the spinal cord in the RIPC/no DMTU group were decreased at 24 h after reperfusion ($P = 0.002$ *vs.* preischemic level). (*C*) Malondialdehyde content in the spinal cord was significantly increased at 6 and 24 h after reperfusion in all the four groups ($P = 0.001$ *vs.* preischemic level). The spinal cord malondialdehyde content in the RIPC/no DMTU group was significantly lower than that in the other three groups at 6 h after reperfusion ($P = 0.001$ *vs.* no RIPC/no DMTU, no RIPC/DMTU, and RIPC + DMTU). * $P < 0.01$ *versus* no RIPC/no DMTU, # $P < 0.01$ *versus* preischemic state. Data are presented as mean ± SD. No RIPC/No DMTU = animal that received sham pretreatment before 20 min spinal cord ischemia; RIPC/No DMTU = animal that received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 min before spinal cord ischemia; No RIPC/DMTU = animal that received sham pretreatment and dimethylthiourea administration (1 h before sham pretreatment) before 20 min spinal cord ischemia; RIPC+DMTU = animal that received RIPC and dimethylthiourea administration (1 h before pretreatment) before 20 min spinal cord ischemia. CAT = catalase; DMTU = dimethylthiourea; MDA = malondialdehyde; RIPC = remote ischemic preconditioning; SOD = superoxide dismutase; Pre = Pre RIPC; RIPC 20 min = 20 min after RIPC; R 6 h = 6 h after reperfusion; R 24 h = 24 h after reperfusion.

Table 3. Physiologic Variables during Preischemic and Postischemic State (n = 6)

	PAP, mmHg	DAP, mmHg	HR, beats/min	T, °C	Pa_{O_2}, mmHg	Pa_{CO_2}, mmHg	PH	G_s, mM
Preischemia								
Control	74 ± 3	95 ± 4	260 ± 6	38.1 ± 0.2	218 ± 33	37 ± 6	7.36 ± 0.03	6.4 ± 0.5
RIPC	76 ± 4	95 ± 5	255 ± 8	38.3 ± 0.5	227 ± 45	38 ± 5	7.38 ± 0.04	5.3 ± 0.4
HEX1	85 ± 3	93 ± 3	256 ± 7	38.4 ± 0.5	215 ± 23	36 ± 5	7.39 ± 0.05	6.1 ± 2.1
HEX2	76 ± 3	94 ± 3	259 ± 9	38.4 ± 0.3	226 ± 67	38 ± 5	7.36 ± 0.01	6.0 ± 0.5
HEX3	77 ± 3	96 ± 3	255 ± 9	38.5 ± 0.2	218 ± 55	37 ± 4	7.37 ± 0.02	5.7 ± 0.4
Ischemia 10 min								
Control	77 ± 4	9 ± 0	270 ± 13	38.2 ± 0.1	230 ± 40	40 ± 4	7.40 ± 0.05	5.6 ± 0.3
RIPC	79 ± 5	9 ± 0	269 ± 15	38.5 ± 0.4	217 ± 35	37 ± 3	7.35 ± 0.06	5.9 ± 0.6
HEX1	88 ± 7	8 ± 1	266 ± 17	38.4 ± 0.5	225 ± 45	41 ± 4	7.39 ± 0.05	6.2 ± 0.5
HEX2	79 ± 9	9 ± 0	271 ± 14	38.7 ± 0.5	231 ± 45	38 ± 3	7.37 ± 0.04	6.4 ± 0.6
HEX3	84 ± 3	9 ± 0	270 ± 12	38.6 ± 0.3	229 ± 52	37 ± 6	7.36 ± 0.03	5.9 ± 0.4
Reperfusion 10 min								
Control	74 ± 2	70 ± 3	280 ± 22	37.9 ± 0.5	236 ± 34	39 ± 3	7.41 ± 0.04	6.1 ± 0.4
RIPC	75 ± 5	75 ± 5	273 ± 21	38.0 ± 0.4	228 ± 56	38 ± 3	7.39 ± 0.05	5.5 ± 0.6
HEX1	77 ± 4	81 ± 4	274 ± 17	38.1 ± 0.3	217 ± 46	37 ± 3	7.42 ± 0.07	6.2 ± 0.5
HEX2	76 ± 4	76 ± 5	271 ± 16	38.3 ± 0.5	237 ± 54	39 ± 4	7.38 ± 0.03	5.8 ± 0.4
HEX3	71 ± 6	78 ± 4	269 ± 14	38.4 ± 0.4	217 ± 35	37 ± 3	7.39 ± 0.06	5.9 ± 0.5

Data are presented as mean ± SD.

Control = animals that received sham pretreatment before 20min spinal cord ischemia; DAP = distal blood pressure; G_s = serum glucose concentration; HEX1 = animals that received RIPC and hexamethonium (HEX) preadministration (30 min before RIPC) 20 min before spinal cord ischemia; HEX2 = animals that received RIPC and hexamethonium (HEX) preadministration (15 min before RIPC) 20 min before spinal cord ischemia; HEX3 = animals that received RIPC and hexamethonium (HEX) preadministration (at onset of RIPC) 20 min before spinal cord ischemia; HR = heart rate; MAP = mean arterial pressure; Pa_{CO_2} = arterial carbon dioxide tension; Pa_{O_2} = arterial oxygen tension; PAP = proximal blood pressure; RIPC = remote ischemic preconditioning, animals that received 2 cycles of occlusion/reperfusion of bilateral femoral arteries for 10 min/10-min interval 30 min before spinal cord ischemia; T = rectal temperature.

citatory amino acids, heat shock protein, and free radicals are all suggested to play important roles in spinal cord ischemic injury.[19,20] However, ameliorative measures, including hypothermia, calcium channel blockers, and free-radical scavengers,[3,21,22] are still inadequate.

IPC has been shown to provide spinal cord protection from ischemic injury.[3,23] However, exposing spinal cord to a brief period of ischemia before an anticipated ischemic event does not seem to be clinically relevant. A series of studies have found that the ischemic tolerance occurred not only within the same piece of tissue but also between different regions and different organs. This phenomenon, which is known as RIPC, has previously been demonstrated in the heart and other organs.[11,24] Recently, some studies have shown that RIPC within a distant organ, such as the kidney, alone or in combination with direct IPC, can induce the ischemic tolerance against spinal cord from ischemic injury.[25]

Table 4. Neurologic Function Scores after Spinal Cord Ischemia

	6 h	24 h	48 h
Control	1 (0–2)	0 (0–1)	0 (0–1)
RIPC	3 (3–4)*	4 (3–4)*	4 (3–4)*
HEX1	3 (2–4)*	4 (3–4)*	4 (3–4)*
HEX2	3 (2–4)*	3 (2–4)*	3 (2–4)*
HEX3	3 (3–4)*	3 (2–4)*	4 (3–4)*

Data are presented as median (range).

* $P = 0.002$ *versus* control.

HEX = hexamethonium; RIPC = remote ischemic preconditioning.

This study confirmed that two sequential brief limb ischemia–reperfusion episodes (remote preconditioning) applied before aortic occlusion can result in significant protection of the spinal cord from ischemic injury. Neurologic outcome in the RIPC/no DMTU group was better than that observed in the ischemia–reperfusion-alone group. This is the first report to demonstrate that a minor ischemic injury, limb RIPC, can induce ischemic tolerance in the spinal cord of rabbits. Because it is easier and safer to perform a brief ischemia on a limb or an arm than on the spinal cord itself before the surgery, this finding has significant clinical applications.

We have previously speculated that some humoral mediators, such as reactive oxygen species, and neural pathway are involved in the induction of ischemic tolerance on spinal cord by RIPC. To test this hypothesis, a reactive oxygen species scavenger, DMTU, was preadministered before RIPC, and the oxidative enzymes (SOD and catalase) in the serum were measured before and after RIPC. Because a direct measurement of liberated free-radical species is limited by their instability, the level of malondialdehyde, a stable product of oxidative degradation of polyunsaturated fatty acids, was adopted as a measure of free-radical formation in this study. The results show that limb RIPC slightly increased the serum malondialdehyde levels and significantly increased the activities of SOD and catalase in the serum of rabbits. We also found that the activity of SOD and catalase in spinal

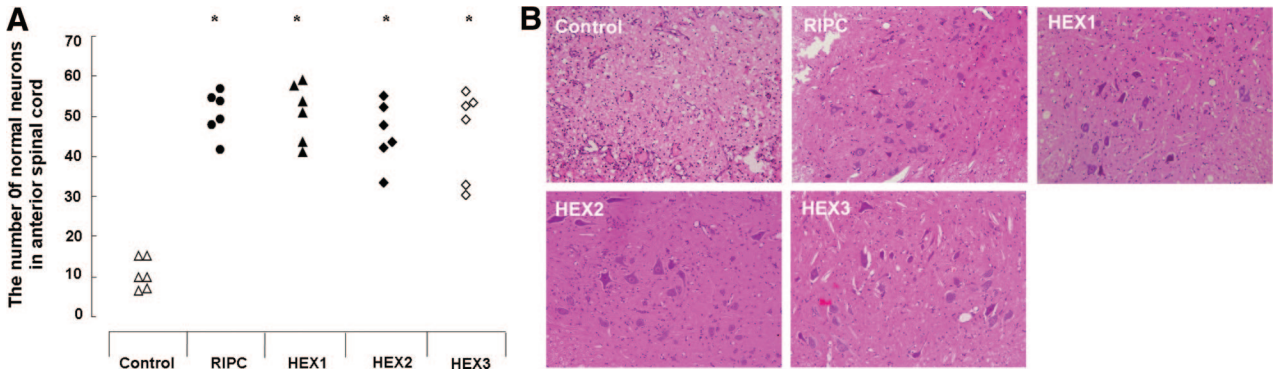

Fig. 5. (*A*) Experiment 2: histopathologic outcome in each animal of the four groups at 48 h after reperfusion. The symbols *filled circle*, *filled triangle*, *open triangle*, *filled diamond*, and *open diamond* represent animals in the control, RIPC, HEX1, HEX2, and HEX3 groups, respectively. (*B*) Representative photomicrographs of lumbar spinal cord sections (L5) at 48 h after reperfusion. Section from a rabbit rated as Tarlov score 0 in the control groups showed severe neuronal damage, as evidenced by disappearance of most of normal motor neurons in the anterior spinal cord and extensive vacuolation of gray matter. Ischemic neurons were identified by cytoplasmic eosinophils with loss of Nissl substance and by the presence of pyknotic homogenous nuclei. Sections from a rabbit rated as Tarlov score 3–4 in the RIPC, HEX1, HEX2, and HEX3 groups showed a number of normal motor neurons in the same area (hematoxylin and eosin staining, magnification 200×). Control, animals received 20-min spinal cord ischemia but were not subjected to RIPC or drug treatment. RIPC = animals that received two cycles of occlusion/reperfusion of bilateral femoral arteries for 10-/10-min interval 30 min before spinal cord ischemia; HEX1 = animals that received RIPC and hexamethonium preadministration (30 min before RIPC) before 20 min spinal cord ischemia; HEX2 = animals that received RIPC and hexamethonium preadministration (15 min before RIPC) before 20 min spinal cord ischemia; HEX3 = animals that received RIPC and hexamethonium preadministration (at onset of RIPC) before 20 min spinal cord ischemia; RIPC = remote ischemic preconditioning; HEX = hexamethonium. * $P < 0.01$ *versus* control.

cord were increased after limb RIPC. However, the malondialdehyde content in spinal cord was unchanged after limb RIPC. The increase in malondialdehyde levels in serum after RIPC suggests that circulating reactive oxygen species is a crucial factor in upregulating the activity of antioxidant enzymes both in the serum and in the spinal cord. Because RIPC stimulates reactive oxygen species production, it is reasonable to imagine that the increased level of reactive oxygen species stimulates the induction of the activity of antioxidant enzymes. We also found that pretreatment with DMTU 1 h before RIPC attenuated the protective effects of RIPC on spinal cord ischemia. The increase in antioxidant enzyme activity induced by RIPC was completely abolished by preadministration of DMTU, in accordance with the neurologic function and histopathologic outcomes. In experiment 2, HEX, a ganglion blocker, was preadministered before RIPC. HEX did not block the protective effect of RIPC on spinal cord ischemic injury. These results indicate that humoral factors such as reactive oxygen species, rather than the neural pathway, mediate the ischemic tolerance of RIPC in spinal cord ischemia.

Although they have been studied extensively in the context of myocardial ischemia,[11,26] the protective mechanisms of RIPC on spinal cord ischemia are still unclear. The protection of IPC is receptor mediated where extracellular signals trigger cell surface receptors, which further activate intracellular second messengers and effectors.[27] Previous studies suggest that a number of substances, including adenosine, bradykinin, reactive oxygen species, and others, are involved in triggering IPC. RIPC might exert its protective effect against ischemia on spinal cord through these substances in a humoral pathway. The results of this study suggest that one of the possible triggering substances, reactive oxygen species, mediate the protective effect of RIPC on spinal cord ischemia in rabbits. We assume that brief limb ischemia produced a great deal of reactive oxygen species locally and the reactive oxygen species were circulated to remote organs, including spinal cord. The increase of reactive oxygen species in serum stimulates the activities of the antioxidant enzymes in the serum and spinal cord.

Our results are in accordance with previous studies that suggest the role of antioxidant enzymes in the formation of the ischemic tolerance induced by various ischemic and non-IPC means. Toyoda *et al.* [28] have found that occlusion of the middle cerebral artery for 20 min, 24 h in advance, significantly increased the activity of the antioxidant enzyme SOD and significantly reduced the cerebral infarction volume. Our previous studies have also demonstrated that spinal cord ischemic tolerance induced by hyperbaric oxygen preconditioning or inhaled anesthetics is triggered by an initial oxidative stress and is associated with an increase in antioxidant enzyme activities as a key mediator in this neuroprotective effect.[12,29] The spinal cord ischemic tolerance induced by hyperbaric oxygen preconditioning was attenuated when a free-radical scavenger was administered before each preconditioning. In another study, lipopolysaccharide-induced ischemic tolerance in the brain parallels the lipopolysaccharide-induced increase in SOD synthesis in the organ.[30]

Several studies have used HEX as a probe to investigate the role of the neurogenic pathway in RIPC against ischemic injury, but the results available are inconsistent. It was reported that a brief mesenteric artery occlusion in the rat preconditioned the myocardium against ischemia, and this antiinfarction effect of RIPC was blocked by HEX pretreatment (20 mg/kg, intravenously).[14] In addition, intramesenteric infusion of bradykinin mimicked the protective effect of

RIPC in the rat, and this protective effect was abolished by pretreatment with HEX (20 mg/kg, intravenously).[31] These observations suggest that the neuronal pathway may play an important role in RIPC of myocardium against infarction. However, other studies reported that the same dose of HEX (20 mg/kg, intravenously) did not attenuate the myocardial infarct-protective effect of remote IPC by 25-min occlusion and reperfusion of the mesentery artery[32] or 15-min occlusion and reperfusion of the infrarenal aorta in the rat.[15] In previous studies, HEX was administered at different times (15 or 2 min before RIPC). Does the interval between drug administration and brief remote ischemia affect the outcome of protective effect of RIPC? To exclude the influence of different time intervals between the HEX administration and RIPC on the results, we administered HEX at different time course before RIPC. The results of our current study show that HEX, when used 30, 15, and 0 min before RIPC, could not attenuate the protective effects induced by limb RIPC. As reported previously,[33] a marked decrease in arterial pressure and a slight increase in heart rate were observed with the administration of HEX in this study. After the fluid infusion and all the procedures were finished, the arterial blood pressure and heart rate returned to baseline before spinal cord ischemia was induced. According to our current findings, a neuronal signal transmission from the remote area, such as limb, to the spinal cord can be excluded with certainty. The humoral factor must be responsible for the remote protection.

In conclusion, our study has demonstrated that limb RIPC is an effective way of reducing spinal cord ischemic injury in a rabbit model. It has also been shown that limb RIPC (ischemia–reperfusion to 10/10 min, two cycles) upregulated the activity of circulating catalase and SOD in the spinal cord and thereby protected the spinal cord against ischemia–reperfusion damage. DMTU, a potent free-radical scavenger, abolished the increase in catalase and SOD activity in the spinal cord after limb RIPC and thus blocked the neuroprotective effect of limb RIPC. These results suggest that reactive oxygen species generated by RIPC triggers the increase of antioxidant enzyme activity both in circulation and in the spinal cord, which scavenge the reactive oxygen species and protect spinal cord from ischemia–reperfusion injury. The neuronal signal pathway is not included in the induction of ischemic tolerance by RIPC.

The authors thank Stephen Brickley, B.Sc., Ph.D. (Senior Lecturer in Neuroscience, Biophysics Section, Imperial College London, London, United Kingdom), for reviewing the manuscript.

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