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Delayed preconditioning effect of isoflurane on spinal cord ischemia in rats

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ABSTRACT

Paraplegia is one of the most common complications following aortic aneurysmal surgery. This study was designed to determine if isoflurane-induced delayed preconditioning is mediated by nuclear factor κB (NF-κB) in the rat spinal cord. The animals were divided into four groups: the control group, the pyrrolidinedithio carbamate (PDTC, an NF-κB inhibitor)-treated group, the isoflurane-treated group, and the PDTC/isoflurane-treated group. In the PDTC-treated groups, 2% 100 mg/kg PDTC was administered intraperitoneally at 1 h before operation and at 24 h and 48 h after reperfusion. The rats in the isoflurane-treated groups received 30 min inhalation of 2.8% isoflurane at 24 h before spinal cord ischemia. Pretreatment with NF-κB inhibitor significantly reduced NF-κB expression and the number of intact motor neurons when compared to the control group. Preconditioning with isoflurane increased the number of normal motor neurons, whereas pretreatment with both PDTC and isoflurane significantly decreased them, compared to the isoflurane-treated group. Isoflurane-induced delayed preconditioning on spinal cord ischemia improved histopathological outcomes. This neuroprotective effect of isoflurane preconditioning on spinal cord ischemia is associated with NF-κB expression.

Paraplegia remains a devastating complication following descending and thoracoabdominal aortic operations. Various strategies have been developed to increase spinal cord tolerance to ischemia and minimize the incidence of neurological complications after aortic surgery [9,22,26,28].

Ischemic preconditioning is a phenomenon whereby a brief period of nonlethal ischemia increases the tolerance of the tissue to a subsequent lethal ischemia. Ischemic preconditioning has two distinct phases: an early phase, which lasts from a few minutes to 2–3 h, and a delayed phase, which develops after the preconditioning stimulus and lasts for several days [16,24]. Isoflurane is a clinically and widely used volatile anesthetic; multiple studies have shown that preconditioning with isoflurane mimics the protective effects of early and delayed ischemic preconditioning in the heart [10,25] and brain [29,31]. Recently, some studies have reported that both early- and late-phase preconditioning with isoflurane exert a neuroprotective effect against spinal cord ischemia [18,20].

Nuclear factor κB (NF-κB) is a redox-sensitive transcription factor regulating a battery of inflammatory genes such as inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase. Activation of NF-κB is a key event in preconditioning-induced neuroprotection [3,19]. However, in brain injury and spinal cord injury models, NF-κB is highly activated, indicating that NF-κB has a critical function of central nervous system (CNS) pathophysiology [1,2,17,21]. Therefore, the roles of the biological actions of NF-κB after spinal cord ischemia are still considered controversial.

The present study was designed to determine whether isoflurane produces delayed preconditioning against spinal cord ischemic injury and, if so, whether the process is mediated by NF-κB by using pyrrolidinedithio carbamate (PDTC), an NF-κB inhibitor, in the ventral horn of the spinal cords of rats.

Male Sprague–Dawley rats weighing 200 ± 10 g (8 weeks in age) were obtained from a commercial breeder (Charles River Technology, Orient Co., Seoul, Korea). The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. The animals were housed in conditions with controlled temperature (20 ± 2 °C) and lighting (from 07:00 h to 19:00 h); they were supplied with food and water ad libitum.

The animals were divided into four groups (n = 6 in each group): the control group, the PDTC-treated group, the isoflurane-treated group, and the PDTC/isoflurane-treated group. The PDTC-treated groups were administered an intraperitoneal injection of 2% 100 mg/kg PDTC (Sigma, St. Louis, MO) at 1 h before operation and at 24 h and 48 h after reperfusion. The rats in the isoflurane-treated

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Table 1
Schematic illustration of experimental protocols

Group	1 day before occlusion	1 h before occlusion	40 min			24 h after reperfusion	48 h after reperfusion
Control			Surgical preparation	Occlusion	Reperfusion		
PDTC		2% PDTC	Surgical preparation	Occlusion	Reperfusion	2% PDTC	2% PDTC
ISO	ISO 2 MAC		Surgical preparation	Occlusion	Reperfusion		
ISO/PDTC	ISO 2 MAC	2% PDTC	Surgical preparation	Occlusion	Reperfusion	2% PDTC	2% PDTC

Control: animals that only underwent spinal cord ischemia without pretreatment; PDTC: animals that received an intraperitoneal injection of 2% 100 mg/kg PDTC at 1 h before operation and at 24 h and 48 h after reperfusion; ISO: animals that received a 30-min inhalation of 2.0 MAC isoflurane at 24 h before spinal cord ischemia; ISO/PDTC: animals that received a 30-min inhalation of 2.0 MAC isoflurane at 24 h before spinal cord ischemia and an intraperitoneal injection of 2% 100 mg/kg PDTC at 1 h before operation and at 24 h and 48 h after reperfusion. ISO 2 MAC: 30 min inhalation of 2.8% isoflurane; 2% PDTC: intraperitoneal injection of 2% 100 mg/kg PDTC. PDTC: pyrrolidinedithio carbamate; MAC: minimum alveolar concentration.

groups received a 30-min inhalation of 2.8% (2.0 MAC) isoflurane (Minrad Inc., Buffalo, NY) at 24 h before spinal cord ischemia as a method of isoflurane preconditioning. The animals in the control group only underwent spinal cord ischemia without pretreatment. MAC is minimum alveolar concentration as a standard measure of the potency of inhaled anesthetics, and it is defined as the equilibrium end-tidal anesthetic concentration expressed as a fraction of 1 atm that prevents movement in response to surgical skin incision in 50% of human subjects. The experimental design is illustrated in Table 1.

Prolonged ischemia was induced based on the method previously described by Zhou et al. [32]. Briefly, the animals were first anesthetized with an intramuscular injection of Zoletil 50® (50 mg/kg; Virbac Laboratories, Carros, France). The peritoneal cavity was then exposed through a vertical midline incision at the upper abdomen. The abdominal aorta was exposed and isolated. Abdominal aorta occlusion was induced by placing a microvascular clamp around the aorta at the level just distal to the left renal artery. Successful arterial occlusion was indicated by the immediate cessation of pulse in the distal artery and a pale appearance in the hind paws of the animals. The microvascular clamp was released after 40 min and the abdominal incision was closed in two layers with silk suture. All animals were allowed to recover postoperatively in each cage, at 25 °C.

All animals were closely monitored after the spinal cord ischemia operation. Neurological functions in each group were assessed at 3 h and 6 h after reperfusion. Hindlimb motor functions were graded using Tarlov scores, in which 0 indicates no movement of hindlimbs, 1 indicates perceptible movements of hindlimb joints, 2 indicates good joint movement but inability to stand, 3 indicates the ability to stand and walk, and 4 indicates a complete recovery. The neurological examinations were done in a blind fashion [12].

For spinal cord tissue preparation, the animals were sacrificed 72 h after reperfusion. In brief, the animals were fully anesthetized with Zoletil 50® (50 mg/kg, i.m.), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde (PFA) in a 100-mM phosphate buffer (PB, pH 7.4). Following perfusion, the entire lumbar segments of the spinal cord (L1–L6) were dissected and removed. The spinal tissues were postfixed for 24 h in the same fixative and then finally kept overnight in the phosphate buffer (pH 7.4) containing 15% sucrose, at 4 °C. Transverse sections of 20 μm thickness for histochemistry and immunohistochemistry and 10 μm thickness for hematoxylin–eosin (HE) staining were made using a freezing microtome (Leica, Nussloch, Germany).

Spinal cords in all groups were excised for histopathological evaluation at 72 h after reperfusion. The sections of lumbar spinal cord (L3–L5) were stained with HE for light microscopic examination, in order to analyze the degree of ischemic cell injury. Intact motor neurons in the ventral horns of the spinal cords (anterior to a transverse line drawn through the central canal) were counted at 400× magnification in each group.

The HE staining took place as follows. Briefly, the slides were dipped into Mayer's hematoxylin for 30 s, and then rinsed with tap water until clear; they were then dipped in eosin for 30 s and again rinsed with water. The slides were air-dried at room temperature and then dipped twice in 95% ethanol, twice in 100% ethanol, twice in a solution of 50% ethanol and 50% xylene, and then twice in 100% xylene. The coverslips were finally mounted using Permount® (Fisher Scientific, Fair Lawn, NJ).

For visualization of NF-κB expression, NF-κB immunohistochemistry was performed based on the method previously described by Tian et al. [23]. Formalin-fixed sections of spinal cord were cultured in 0.3% H_2O_2 for 20 min to remove endogenous peroxidase, quenched with normal goat serum (Vector Laboratories) for 1 h to block nonspecific binding, and incubated overnight at 4 °C with polyclonal rabbit anti-rat NF-κB p65 against purified recombinant NF-κB. Then, anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxides were added. For visualization, the sections were incubated in 50 mM Tris–HCl (pH 7.6) containing 0.02% DAB and 0.03% H_2O_2 for 5 min, and the sections were finally mounted onto gelatin-coated slides. The slides were air-dried at room temperature and coverslipped with Permount®.

The numbers of motor neurons stained with HE and NF-κB-positive in the ventral gray matter region were surveyed and counted hemilaterally in 10 sections of the L3–L5 segments of spinal cord using a light microscope (Olympus, Tokyo, Japan). Differences were determined by one-way ANOVA, followed by a Duncan *post*

Table 2
Neurological outcomes observed at 3 h, 6 h, and 72 h after reperfusion

Tarlov scale	3 h after reperfusion					6 h after reperfusion					72 h after reperfusion				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Control (n = 6)		1	4	1				1	2	3					6
PDTC (n = 6)		1	5					1	3	2					6
ISO (n = 6)		1	2	3					3	3					6
ISO/PDTC (n = 6)		1	3	2					4	2					6

Control: animals that only underwent spinal cord ischemia without pretreatment; PDTC: animals that received an intraperitoneal injection of 2% 100 mg/kg PDTC at 1 h before operation and at 24 h and 48 h after reperfusion; ISO: animals that received a 30-min inhalation of 2.0 MAC isoflurane at 24 h before spinal cord ischemia; ISO/PDTC: animals that received a 30-min inhalation of 2.0 MAC isoflurane at 24 h before spinal cord ischemia and an intraperitoneal injection of 2% 100 mg/kg PDTC at 1 h before operation and at 24 h and 48 h after reperfusion. PDTC: pyrrolidinedithio carbamate; MAC: minimum alveolar concentration.