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TREK1 activation mediates spinal cord ischemic tolerance induced by isoflurane preconditioning in rats

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ABSTRACT

The aim of this study is to examine the role of one of the two-pore (2P) domain K^+ channels, TREK (TWIK-related K^+ channels, TREK)-1, mediated neuroprotection on spinal cord afforded by isoflurane preconditioning. In Experiment 1, male Sprague-Dawley rats were randomly assigned to control (Con) group, an isoflurane preconditioning (Iso) group, and sham group. Twenty-four hours after the last pretreatment, spinal cord ischemia was induced in Con and Iso groups. Neurobehavioral testing and histopathologic examination were performed after reperfusion. In Experiment 2, the expression of the TREK1 in the spinal cord was assessed by immunohistochemistry, Western blot and real-time polymerase chain reaction. In Experiment 3, Amiloride, a blocker of stretch-sensitive channels, was administered intraperitoneally immediately prior to each isoflurane preconditioning. Iso group showed a significant reductions in motor deficit index as well as increases in the number of normal neurons compared with the Con group. The expression of TREK1 protein and the level of mRNA after ischemia were higher in the rats of the Iso group than those in the Con group. Amiloride pretreatment abolished the protective effects of Iso preconditioning. These finding indicate that isoflurane preconditioning had a neuroprotective effect against spinal cord ischemia reperfusion injury. These effects may be mediated through the TREK1 pathway.

Spinal cord ischemia-reperfusion injury is a catastrophic event that can result in permanent disability or death. This type of spinal cord injury has a high incidence during thoracic and thoracoabdominal aneurism repair surgery. It has been reported that the incidence of paraplegia in thoracoabdominal aneurysm repair surgery and in posterior longitudinal ligament surgery is 16% [27] and 11.8% [22], respectively. Although several neuroprotective means and agents can decrease the risk of postoperative paraplegia, the prevention of spinal cord ischemic injury remains a great concern during thoracic aorta and spine surgery [15].

Recently, the effects of ischemic preconditioning [16] have also been observed after treatment with volatile anesthetics. In our laboratory, Xiong et al. [25] have shown that repeated brief isoflurane anesthesia induced ischemic tolerance in the brain in rats. In parallel, several animal studies have shown that volatile anesthetics significantly decrease spinal cord ischemic injury and that the inhaled anesthetics may be able to provide neuroprotection [20,3]. In addition, mounting clinical evidence has shown that inhaled anesthetic pretreatment during surgery may prevent or attenuate cardiac and hepatic ischemic injury [1,9]. Therefore, volatile anesthetic preconditioning may be a more clinically applicable means of preventing spinal cord ischemic injury in the future.

In addition, the mechanisms of volatile anesthetic-induced preconditioning are complex and biphasic. Since 1982 [11], the activation of potassium channels has long been thought to play an important role in the actions of general anesthetics [17] and preconditioning [4]. Two-pore-domain K^+ (K2P) channels are a diverse and highly regulated super-family of background potassium channels that can be activated by the anesthetic gases. TREK1 channel, which represent one member of this superfamily, has been shown to be markedly activated by clinically relevant concentrations of volatile general anesthetics such as isoflurane [5].

Thus, in the present study, we investigated whether the robust neuroprotection afforded by isoflurane-induced preconditioning could be attributed to TREK1 channels, which may prove to be a promising target for spinal cord neuroprotection.

In Experiment 1, 30 male Sprague-Dawley rats (300–350 g) were randomly assigned to 3 groups ($n = 10$ in each): control (Con), isoflurane (Iso) and Sham. Rats in the Con group received 100% oxygen

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1 h/d for 5 days; rats in the Iso group received 1.4% isoflurane in oxygen 1 h/d for 5 days; and rats in the Sham group received the same operation as the two previous groups but without preconditioning or spinal cord ischemia. The concentration of isoflurane and oxygen was measured constantly using a monitor for volatile anesthetics (Philips IntelliVue MP60, Netherlands) during preconditioning. After preconditioning, rats were allowed to recover freely in room air. Twenty-four hours after the last preconditioning, spinal cord ischemia was induced for 12 min followed by 48 h of reperfusion in Iso and Con groups. Rats were assessed using neurobehavioral tests 24 and 48 h after reperfusion. All animals were sacrificed at 48 h after reperfusion, and histopathologic examinations were performed to calculate the number of normal neurons in anterior spinal cord.

Spinal cord ischemia models and neurologic and histopathologic evaluations have been described in detail previously [21,23]. Briefly, a 2F Fogarty catheter was inserted from the femoral artery into the thoracic aorta until the tip of the catheter reached the left subclavian artery. The balloon was inflated with 0.05 ml of saline and the aortic occlusion was confirmed by a reduction of the DAP. A blood-collecting circuit (37.5 °C) was connected to the carotid artery and positioned at a height of 60 cm (45 mm Hg) in order to maintain systemic hypotension (45 mm Hg) during the occlusion. At the end of ischemia, the balloon was deflated, and the blood was reinfused within 60 s. When the blood was reinfused, protamine sulfate (4 mg) was administered subcutaneously. The heart rate, proximal arterial blood pressure (PAP), distal arterial blood pressure (DAP), and temperature were monitored. Blood gas analysis was performed during the operation. The animals were neurologically assessed 24 h and 48 h after reperfusion by an investigator who was unaware of the animals' groupings as reported. Motor deficit index (MDI) was scored from 0 to 6, where 0 is normal and 6 represents the most serious neural injury. After completion of the evaluation at 48 h, animals were deeply anesthetized with sodium pentobarbital (60 mg/kg). Transcardiac perfusion and fixation were performed with 200 mL of 0.9% saline solution followed by 250 mL of 4% paraformaldehyde. The lumbar spinal cord was removed and refrigerated in the same fixative overnight. Three representative sections were taken from the L4 to L6 segments and stained with hematoxylin and eosin. The numbers of viable motor neurons were counted. The averages were used and compared among groups [2].

In Experiment 2, 35 rats were divided into 3 groups: a sham group ($n = 5$), an Iso group and a control group ($n = 15$ in each group). The surgery and preconditioning procedures were the same as those in Experiment 1. Animals were correspondingly sacrificed at 4 h, 24 h, and 48 h after reperfusion to assess the expression of TREK1 in the spinal cord using immunohistochemistry, Western blotting and real-time PCR ($n = 10$ in each time point). In Experiment 3, 40 male Sprague-Dawley rats were randomly assigned to 4 groups ($n = 10$): control (Con), isoflurane (Iso), Iso + AMI (amiloride), and AMI groups. The surgery and preconditioning procedures in Con and Iso group were the same as those in Experiment 1. Amiloride (2.5 mg/kg) was intraperitoneally administered immediately prior to each isoflurane preconditioning in the Iso + AMI groups. Animals in the AMI group received amiloride only. The procedure was the same as Experiment 1.

Immunohistochemistry was performed on cryosections as described previously [23]. The tissues of L4–L6 were cut into 40 μm sections in a cryostat (CM1900, Leica). The sections were kept in 3% hydrogen peroxide for 15 min to quench the endogenous peroxidase activity and then transferred to 5% rabbit serum for 30 min for blocking. They were then incubated with primary antibodies (1:50 dilution, Santa Cruz Biotechnology) for 2 h at 37 °C and washed in PBST three times for 10 min. Next, sections were incubated with TRITC-conjugated secondary antibodies (1:100 dilution, rabbit anti-goat IgG, Santa Cruz Biotechnology) at room temperature for 2 h with gentle agitation. Sections were then transferred onto glass slides and covers lipped with Mowiol mounting medium and fluorescence imaging was performed using a Olympus SZ61 Stereo microscope (Olympus Corporation, Tokyo, Japan).

Tissue samples were homogenized using a whole protein extract kit (Millipore Corporation, Bil-lerica, MA) with a glass tissue grinder. Protein content was determined using the BCA protein assay kit (Millipore Corporation). Equal amounts of protein per lane (50 μg) were loaded onto an 8% (v/v) polyacrylamide gel and separated by electrophoresis at 120 V for 60 min. Proteins were then transferred to nitrocellulose (Bio-Rad Laboratories, Inc., Hercules, CA) at 20 V for 50 min and the membrane was blocked with 10% (w/v) nonfat dry milk and 0.5% (v/v) Tween-20 in Tris-buffered saline. The membrane was incubated with two different antibodies overnight at 4 °C, namely rabbit anti TREK antibody (ab83932, 1:1,000; Abcam). The membrane was incubated with a horseradish peroxidase conjugated secondary antibody (anti-rabbit IgG, 1:2000, Vector Laboratories) for 60 min at 37 °C. The specific protein bands were visualized using the standard chemical luminescence method. Image analysis was performed with the assistance of a computer.

Total RNA was extracted from spinal cord tissue using Trizol reagent (Invitrogen Technology) following *the* manufacture's protocol. The concentration of RNA was quantified from the optical density measured at 260 nm by ultraviolet spectrophotometry. Forward and reverse primers used in the present study: TGACCTCAGACAGTCGGTAT/CAAGCCTGCTATACCTCG, β-actin GTGCCCA TCTATGAGGGTTACGCG/GGAACCGCTCATTGCCGATAGTG. Equal amounts of RNA (500 ng) from each sample were reverse transcribed in a volume of 10 μl to produce cDNA using Takara RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc.). Reverse transcription reactions were performed at 37 °C for 15 min and 85 °C for 5 s. Standard curves plotting the threshold amplification cycle number values against input quantity for each gene were constructed using fivefold serial dilutions of RT product. β-Actin served as an endogenous internal standard control. SYBR Green I-based detection was carried out on a real-time PCR instrument (ABI PRISM 7300) with thermal cycler conditions of: 95 °C for 30 s, followed by 45 cycles (95 °C for 10 s and 62 °C for 31 s). All experiments were repeated twice and, in each experiment, samples were assayed in duplicate. Data were expressed as a ratio: relative quantity of TREK1 mRNA/relative quantity of β-actin mRNA, respectively.

Blood pressure, blood gases, plasma glucose, Western blot analysis, RT-PCR data and number of normal neurons in the rostral cord were analyzed using a one-way analysis of variance, followed by a Student–Newman–Keuls test for multiple comparisons and are presented as the mean ± SD. The scores for hind limb motor function are reported as the median, and were compared using a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney *U*-test. A $P < 0.05$ was considered to be statistically significant.

In Experiment 1, physiologic variables were similar in all groups, and no statistically significant differences were noted (data are not shown). All animals survived until the final neurologic behavior assessment at 48 h after reperfusion. The neurological function scores at 48 h after reperfusion for the 3 groups are shown in Fig. 1. The hind limb MDI of the Iso group was significantly lower than that of the Con group ($P = 0.001$ vs. Con). Rats in the sham group demonstrated completely normal motor function (MDI = 0) (Fig. 1). The number of normal neurons in the Iso group was significantly greater than that of the con group ($P = 0.018$) (Fig. 2B). No injured neuron were found in the sham group (Fig. 2A).

In Experiment 2,immunohistochemical staining of TREK 1 in the lumber spinal cord region is shown in Fig. 3A. In both groups, more TREK 1-stained neurons were detected 4 h, 24 h and 48 h after spinal cord reperfusion compared with the sham group. Isoflurane