

Isoflurane Preconditioning Induces Neuroprotection by Up-Regulation of TREK1 in a Rat Model of Spinal Cord Ischemic Injury

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Abstract

This study aimed to explore the neuroprotection and mechanism of isoflurane on rats with spinal cord ischemic injury. Total 40 adult male Sprague-Dawley rats were divided into the four groups (n=10). Group A was sham-operation group; group B was ischemia group; group C was isoflurane preconditioning group; group D was isoflurane preconditioning followed by ischemia treatment group. Then the expressions of TWIK-related K⁺ channel 1 (TREK1) in the four groups were detected by immunofluorescent assay, real time-polymerase chain reactions (RT-PCR) and western blot. The primary neurons of rats were isolated and cultured under normal and hypoxic conditions. Besides, the neurons under two conditions were transfected with green fluorescent protein (GFP)-TREK1 and lentiviral to overexpress and silence TREK1. Additionally, the neurons were treated with isoflurane or not. Then caspase-3 activity and cell cycle of neurons under normal and hypoxic conditions were detected. Furthermore, nicotinamide adenine dinucleotide hydrate (NADH) was detected using NAD⁺/NADH quantification colorimetric kit. Results showed that the mRNA and protein expressions of TREK1 increased significantly in group C and D. In neurons, when TREK1 silenced, isoflurane treatment improved the caspase-3 activity. In hypoxic condition, the caspase-3 activity and sub-G1 cell percentage significantly increased, however, when TREK1 overexpressed the caspase-3 activity and sub-G1 cell percentage decreased significantly. Furthermore, both isoflurane treatment and overexpression of TREK1 significantly decreased NADH. In conclusion, isoflurane-induced neuroprotection in spinal cord ischemic injury may be associated with the up-regulation of TREK1.

Key Words: Isoflurane, Spinal cord ischemic injury, TREK1, Caspase-3, NADH

INTRODUCTION

Spinal cord ischemia injury, a devastating progressive neurological disorder, can result in permanent disability or death (Yin *et al.*, 2012). It may be related to mechanical injury to spinal cord or neuronal cell death caused by substances released from cells in response to interruption of spinal cord blood flow (Kwon *et al.*, 2004). It is also a complication of surgical repair of thoracoabdominal aortic aneurysm or type B aortic dissection (Coselli *et al.*, 1997; Safi *et al.*, 1998). Various strategies have been proposed to reduce the incidence of spinal cord ischemia injury, however, the risk has not been eliminated.

Some studies have indicated that ischemic preconditioning has protective actions in many organs (Zheng and Zuo, 2004; Wakeno-Takahashi *et al.*, 2005; Mònaco *et al.*, 2007). However, ischemic preconditioning is impractical for clinical prac-

tice (Tong *et al.*, 2014). Yang *et al.* (2011) found that volatile anaesthetics preconditioning could induce neuronal tolerance to ischaemia in rats. Importantly, this kind of preconditioning is less invasive. Isoflurane is a commonly used volatile anesthetic, which has been demonstrated to be neuroprotective against spinal cord ischemia (Zheng and Zuo, 2004; Sang *et al.*, 2006). However, the underlying mechanisms for its neuroprotective effects are unclear. Previous studies have found that the activation of potassium channels may play a critical role in the actions of general anesthetics and preconditioning (Franks and Honoré, 2004; Kitano *et al.*, 2007). TWIK-related K⁺ channel 1 (TREK1) channel, a member of two-pore-domain K⁺ (K2P) channels superfamily, has been shown to be markedly activated by volatile general anesthetics including isoflurane (Gruss *et al.*, 2004). Importantly, the activation of TREK1 mediates neuroprotection (Franks and Honoré, 2004).

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Even so, the potential mechanisms in spinal cord protection by TREK1 activation are not fully understood.

Therefore, in the present study, we established some rat models of spinal cord ischemic injury and investigated the expression of TREK1 after they were preconditioned by isoflurane. Besides, we made TREK1 over-expressed and silenced and then investigated the apoptosis and the production of reduced nicotinamide adenine dinucleotide hydrate (NADH) in neurons. We aimed to study whether the neuroprotection of isoflurane preconditioning was attributed to TREK1.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats, weighing 220-250 g, were used in this study. The animals were synchronized to a 12/12 h light/dark cycle and housed at 20-22°C before the experiment. All animals were allowed free access to laboratory chow and tap water. All experimental procedures were in accordance with the protocols approved by the Ethics Committee for Animal Experimentation.

Total 40 specific pathogen free adult male rats were divided into the four groups (n=10). Group A was sham-operation group; group B was 24 h ischemia group; group C was isoflurane preconditioning group (15 mL/L isoflurane was inhaled by rats and the treatment was performed for five consecutive days and 1 h for each day); group D was isoflurane preconditioning followed by ischemia treatment group. The surgical procedures of ischemia induction were as follows: the rat was anesthetized with 10% chloral hydrate (0.10035 mL/g intraperitoneally). The left external carotid artery (far from heart) was ligated under operating microscope. Then a wedge incision was made on the starting site of external carotid artery stumps, and a fishing line with diameter of 0.25 mm (head-end diameter of 0.3 mm) was inserted into the internal carotid artery from the incision so that the head-end of the fishing line reached the beginning of the middle cerebral artery to block the blood supply of the middle cerebral artery. A successful model of spinal cord ischemic injury developed ipsilateral Horner syndrome and hemiplegia in the contralateral forelimb. The surgical procedures of group A were the same as that of the ischemia group except that the fishing line was not inserted.

Immunofluorescent assay

After depth of anesthesia, the rats in four groups were decapitated. Their brains were rapidly harvested and quick-frozen in isopentane. Then the frozen brains were cut into slices (10 μ m thick) using the freezing microtome (Shenyang Longshou Electronic Equipment Co., Ltd., Shenyang, Liaoning, China) at -20°C. The slices were put on the glass coverslips coated with polylysine and fixed with 4% (v/v) paraformaldehyde in phosphate buffer solution (PBS) for 15 min, and then thoroughly rinsed with 0.01 mol/L PBS, followed by sarcolemmal rupture with Triton X-100 and 10% bovine serum albumin (BSA) blocking for 2 h at room temperature. Primary antibodies used were TREK1 (1:200), glial fibrillary acidic protein (GFAP) (1:200), and neuron-specific nuclear protein (NeuN) (1:200). After incubation overnight at 4°C, the coverslips were washed with 0.01 mol/L PBS and incubated with Cy3-conjugated goat anti-mouse secondary antibodies for 1 h at room temperature.

All antibodies were purchased from Santa Cruz Biotechnology (Texas, USA). Images were captured using an Olympus BX51 fluorescent microscope (Suzhou yi micro-optics, Suzhou, Jiangsu, Chian).

RNA isolation and real time-polymerase chain reactions (RT-PCR)

Total RNAs of the brain tissue slice of rats in four groups above were isolated using trizol reagent (Invitrogen, New York, USA) following the manufacturer's instruction. Five μ g RNA was used to synthesize the first strand of cDNA using SuperScript II RT 200 U/ml (Invitrogen, New York, USA). Forward and reverse primer sequences of TREK1 were TGACCTCAGACAGTCGGTAT/CAAGCCTGCTATACCTCGT. The housekeeping gene β -actin was used as internal control and its primer sequences were GTGCCCATCTATGAGGGT-TACGCG/GGAACCGCTCATTGCCGATGTG. The reaction system (20 μ l) was mixed with 2 \times SYBR Premix Ex Taq 10 μ l, forward and reverse primers (2.5 μ M) 0.8 μ l, cDNA 5 μ l, ddH₂O 4.2 μ l). Conditions of the two-step polymerase chain reaction (PCR) were as follows: 1 cycle at 95°C for 1 min, then 40 cycles at 95°C for 5 s, and 60°C for 20 s. Data were analyzed using 2^{- $\Delta\Delta$ CT} statistical method. Results were presented as CT values and the average CT was calculated for both TREK1 and β -actin, and Δ CT was determined as the ratio of the mean of the triplicate CT values for TREK1 to the mean of the triplicate CT values for β -actin.

Western blot analysis

The brain tissue slice of rats were treated with rapamycin or Torin 1 for 1 h, followed by whole cell lysis in NP-40 lysis buffer (Invitrogen, New York, USA). Whole cell lysates were mixed with 5 \times sodium dodecyl sulfate (SDS) sample buffer to a final concentration of 2 mg/mL, boiled for 5 minutes, and then directly used for immunoblotting. Total 20 mg of whole cell lysates were subjected to 4-12% bis-tris SDS-polyacrylamide gel electrophoresis (Invitrogen, New York, USA), and then transferred to 0.45 mm polyvinylidene difluoride membrane (Millipore, Massachusetts, USA) at 130 mA for 2 h. After blocked in 5% defatted milk dissolved in Tris-buffered saline Tween-20, the membrane was incubated with anti-S6K1 (clone 49D7) (Cell Signaling Technology, Massachusetts, USA), anti-phospho-T389-S6K1 (clone 108D2) (Cell Signaling Technology, Massachusetts, USA), and anti-actin (clone GT239) (GeneTex, Texas, USA). All primary antibodies were diluted at 1: 1000. After rinsing, the membrane was incubated with Horseradish-peroxidase-labeled anti-rabbit secondary antibodies (1: 5000) (Santa Cruz Biotechnology, Texas, USA). After washing, the membrane reacted with enhanced chemiluminescence reagent (Thermo, Massachusetts, USA) for 1 min and developed with medical blue X-ray film (Millipore, Massachusetts, USA) in a dark room.

Primary hippocampal neuron cell cultures

The newborn rats were disinfected with alcohol for 5-10 min and then were decapitated. After the skull was opened, the cerebral hemisphere exposed, and the meninges were removed. Then hippocampus was isolated, sliced and digested with 1 ml 0.25% trypsin for 25-30 min at 37°C. The digested cell debris was moved to 2 ml centrifuge tube with 2 ml nutrient solution (80% dulbecco's modified eagle medium, 10% fetal bovine serum and 10% horse serum) to terminate the digestion. The

cell mass were moved to another centrifuge tube using the fire polishing pipette, then 2 ml nutrient solution was added followed by blowing 20 times. After 5-10 min standing, 1 ml upper cell suspension was transferred, and 1 ml nutrient solution was added, and then blown and transferred as above. The collected cell suspensions were inoculated into cell culture plate. For the normal condition group, cells were incubated in a humidified atmosphere at normal conditions of 20% O₂, 5% CO₂ and 75% N₂ at 37°C. For the hypoxic condition group, cells were incubated in a humidified atmosphere at conditions of 93% N₂, 5% CO₂ and 2% O₂ at 37°C.

Green fluorescent protein (GFP)-TREK1 transfection and siRNA-TREK1 silence

The GFP-TREK1 was transfected into neurons cells (cultured at both normal condition and hypoxic condition) using Lipofectamine® 2000 (Invitrogen, New York, USA) following the manufacturer's protocol.

The inhibition of TREK1 was performed with lentiviral conduction. TREK1 siRNA was designed and purchased from Qiagen (Hilden, German). The target sequence of the TREK1-siRNA used in this study was: 5'-CACGACCATTAATGTTATGAA-3'. The cells were divided into siRNA for TREK-1 group, and siRNA control group. The sequences were cloned into feline immunodeficiency virus based lentivirus expression vector and co-expressed with pPACK packaging system in 293 TN cells. Then lentivirus or siRNAs were delivered to rat hippocampus through an injection on the right side of the brain. siRNA validation was carried out at 24 h by checking the expression of TREK1 by western blot analysis.

Isoflurane exposure

The neurons under two culture conditions above were placed in a Billups-Rothenburg chamber (Billups-Rothenburg, California, USA). The chamber was flushed with 15 mL/L isoflurane for 15 min in humidified air with 5% CO₂ at 37°C. The chamber was then sealed and placed inside a 37°C incubator. The concentrations of O₂, isoflurane and CO₂ in the chamber were monitored using a Datex Capnomac Ultima gas analyzer (Datex Ohmeda, Helsinki, Finland) at 2-h intervals. If there was any change, the chamber was re-flushed. Control experiments were performed in the same manner except that no isoflurane was added when flushing the chamber.

Caspase-3 activity and poly-ADP-ribose polymerase (PARP) cleavage assay

The caspase-3 activity assay (Roche, Basle, Switzerland) was performed according to the manufacturer's instructions. In brief, cells were harvested and lysed for 30 min. After centrifugation, the supernatant was incubated with caspase-3 enzyme reaction system for 60 min and measured at 405 nm. The activity was compared with standard curve and calculated.

The poly-ADP-ribose polymerase (PARP) cleavage in neurons under different conditions were detected using the PARP Cleavage Western Blotting Kit (Enzo Biochem, California, USA) according to the manufacturer's instructions.

Flow cytometry

In order to analyze the impact of TREK1 on cell cycle, flow cytometry was performed with propidium iodide (PI) staining. After lentivirus infection for eight days, different cells were

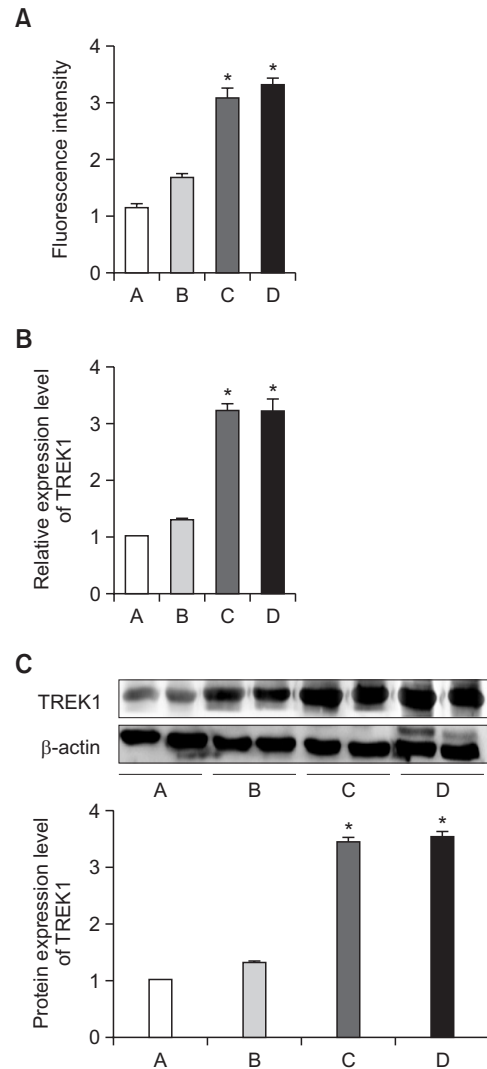


Fig. 1. Expression of TREK1 in four groups detected by immunofluorescent assay (A), real time-polymerase chain reactions (B) and western blot (C). Group A: sham-operation group; group B: ischemia group; group C: isoflurane preconditioning group; group D: isoflurane preconditioning plus ischemia treatment group. Data were presented as mean \pm standard deviation (* $p < 0.05$).

seeded in 6 cm dish at a density of 60000 infected cells per well and cultured in incubator for 40 h. The cells were collected when the concentration reached 80%. After washed with ice-cold PBS, cells were fixed with ice-cold 70% ethanol for 30 min at 4°C, and then resuspended. The suspension was filtered through a 400 meshes and stained with PI in dark for 30 min at 4°C. Tests were carried out independently in three times for each sample using fluorescence activated cell sorter.

NAD⁺/NADH assay

NADH measurement was performed according to the manufacturer's instructions. To detect NADH, NAD needed to be decomposed before the reaction. To decompose NAD, 200 μ l of extracted samples were aliquoted into eppendorf tubes, and were heated to 60°C, lasting for 30 min. The decomposed lysis was reacted and measured at 450 nm. NADH in the

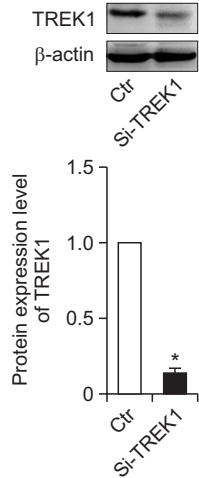


Fig. 2. Interfering efficiency of TREK1 in neurons. Data were presented as mean \pm standard deviation (* p <0.05).

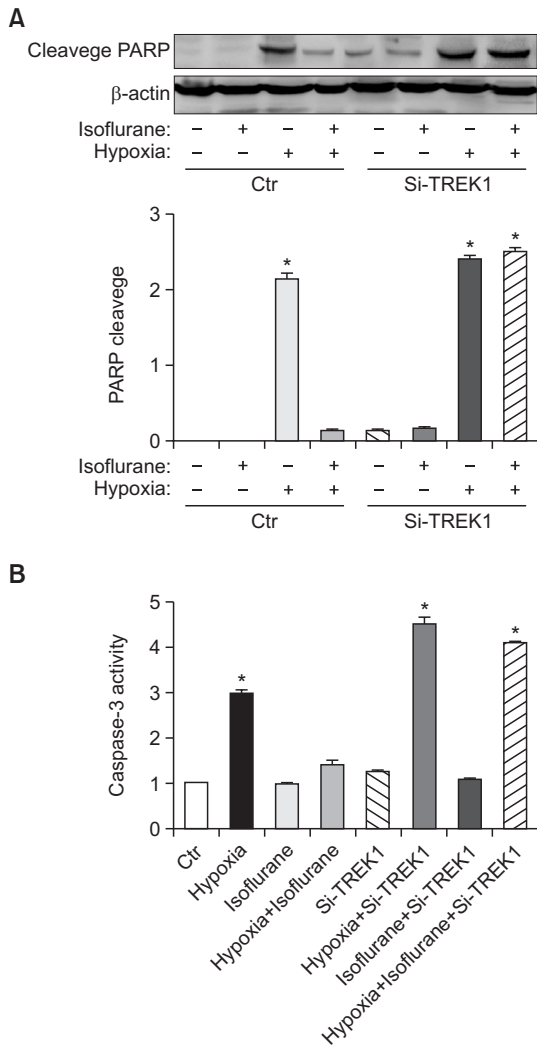


Fig. 3. PARP cleavage (A) and caspase-3 activity (B) when TREK1 was interfered with si-TREK1 in neurons. Data were presented as mean \pm standard deviation (* p <0.05).

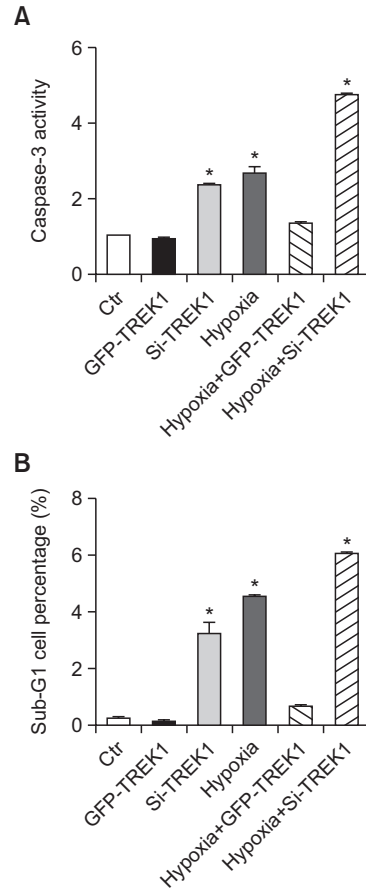


Fig. 4. Caspase-3 activity (A) of neurons and percentage of sub-G1 cells (B) when TREK1 was transfected (GFP-TREK1) or interfered (si-TREK1) under normal and hypoxic conditions. Data were presented as mean \pm standard deviation (* p <0.05).

sample wells was calculated according to the NADH standard curve.

Statistical analysis

This experiment repeated three times. All data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data were expressed as mean \pm standard deviation of three independent experiments. All collected data were firstly tested for the normal distribution using one-sample K-S test. Measurement data were tested by student t-test (for two groups) or one way analysis of variance (ANOVA, for more than three groups). p \leq 0.05 was considered statistically significant.

RESULTS

Expression of TREK1

The fluorescence intensity, mRNA expression and protein expression of TREK1 in four groups were shown in Fig. 1. As shown in Fig. 1A, the fluorescence intensities in group C and D (isoflurane preconditioning) were significantly higher than that in group A and B (p <0.05). Similarly, the mRNA (Fig. 1B) and protein expressions (Fig. 1C) of TREK1 after isoflurane

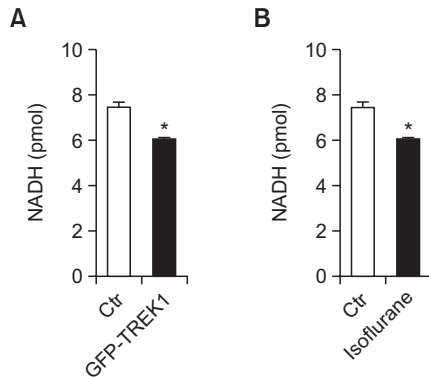


Fig. 5. NADH assay when TREK1 was transfected (GFP-TREK1) (A), and neurons were preconditioned with isoflurane (B). Data were presented as mean \pm standard deviation (* $p < 0.05$).

preconditioning increased significantly compared with that with isoflurane preconditioning ($p < 0.05$).

TREK1 silence

In order to determine the role of TREK1 in neurons, we transfected siRNA-TREK1 to neurons to make TREK1 silence. As shown in Fig. 2, when TREK1 was interfered by lentivirus-mediated siRNA, the expression of TREK1 protein decreased significantly ($p < 0.05$).

For the expression of PARP cleavage, it was high under hypoxic condition regardless of TREK1 silence or not (Fig. 3A). However, when isoflurane was added, PARP cleavage decreased significantly ($p < 0.05$) in control group, but in TREK1 silence group, PARP cleavage did not decrease significantly (Fig. 3A). Similarly, the caspase-3 activity showed similar variation tendency (Fig. 3B). This result indicated that TREK1 may play a key role in the neuroprotection of isoflurane.

Neurons apoptosis assay

In order to further investigate the effect of TREK1 on neurons, the activity of apoptosis-related protein caspase-3 and the percentage of sub-G1 cells with and without TREK1 treatment were detected, and the results were shown in Fig. 4. From Fig. 4, we could find that both the caspase-3 activity and the percentage of sub-G1 cells under hypoxic condition were significantly higher than that under normal condition ($p < 0.05$), indicating that hypoxia may induce apoptosis. However, when TREK1 over-expressed (GFP-TREK1), the caspase-3 activity and percentage of sub-G1 cells decreased significantly under both normal and hypoxic conditions ($p < 0.05$). On the other hand, TREK1 silence (si-TREK1) significantly increased the caspase-3 activity and percentage of sub-G1 cells ($p < 0.05$). The results indicated that TREK1 could decrease neurons apoptosis caused by hypoxia or ischemia.

NADH assay

NADH is a major reducing power of cells in respiratory action. As shown in Fig. 5A, over-expression of TREK1 reduced NADH significantly ($p < 0.05$). Isoflurane preconditioning could also decrease NADH significantly ($p < 0.05$) (Fig. 5B).

DISCUSSION

In this study, the role of TREK1 in the neuroprotective effect induced by isoflurane preconditioning was studied in vitro and in vivo. The present study suggested that isoflurane exposure increased the TREK1 expression in spinal cord ischemic injury rats. The over-expression of TREK1 could decrease apoptosis and NADH in neurons. Tolerance to ischemia in neurons induced by isoflurane preconditioning is reduced by down-regulation of TREK-1 via RNA interference. Findings of this study indicated that TREK1 might play a pivotal role in the induction of the neuroprotective effect by isoflurane preconditioning.

Transient ischemic preconditioning is an effective method to protect neurones from severe ischemic injury, however, ischemic preconditioning is invasive for clinical practice (Dirnagl *et al.*, 2009). More and more studies have found that preconditioning using anaesthetic agents, especially for volatile anaesthetic agents such as isoflurane, is more amenable to induce ischemic tolerance in rabbits and rats (Sang *et al.*, 2006; Liu *et al.*, 2006; Zhang *et al.*, 2010). Nevertheless, the precise mechanisms underlying the improvement of neurological function by isoflurane preconditioning remain unclear. In recent years, K2P channels have received considerable attention in neuroprotection (Tong *et al.*, 2013). K2P channels are a highly regulated superfamily of potassium channels which can be activated by the anesthetic gases (Siegelbaum *et al.*, 1982). As a subfamily of K2P channels, TREK channels are predominantly expressed in the central nervous system (Mirkovic *et al.*, 2012). Study has found that TREK channels can be regulated by various pharmacological agents including volatile anaesthetics (Enyedi and Czirjak, 2015; Franks and Honoré, 2004). In our study, the up-regulation of TREK1 in group C and D was in accordance with the findings above.

Study has found that ischemia contributes to the apoptosis of neurons (Chaitanya and Babu, 2008). Ischemic cell death is often due to the involvement of several apoptogenic proteins (Chaitanya and Babu, 2008). Caspase-3 is a member of the caspase family of 13 aspartate-specific cysteine proteases, which plays an important role in the apoptotic program (Shabbir *et al.*, 2015). Activation of caspase-3 leads to DNA fragmentation (Li *et al.*, 1997). In our study, flow cytometry found that the percentage of sub-G1 cells increased significantly under hypoxic conditions, besides, the caspase-3 activity increased as well. Interestingly, when neurons were transfected with GFP-TREK1, the percentage of sub-G1 cells and caspase-3 activity decreased significantly. When neurons were interfered with siRNA, the percentage of sub-G1 cells and caspase-3 activity increased again. Heurteaux *et al.* (2004) also suggested that TREK1^{-/-} mice had increased sensitivity to ischaemia. Taken together, this result indicated that the over-expression of TREK1 might reduce the apoptosis of neurons to play a role of neuroprotection.

The mitochondrial electron transport chain generates a lot of reactive oxygen species (ROS) which are deleterious during ischemia (Ambrosio *et al.*, 1993). Previous studies have indicated that NADH is one of the primary ROS-forming sites of the mitochondrial electron transport chain (Turrens and Boveris, 1980; Otáhal *et al.*, 2014). Specially, isoflurane has been shown to inhibit the electron transport chain at the level of NADH (Hanley *et al.*, 2002). In our study, isoflurane significantly decreased NADH, furthermore, over-expression of TREK1 also significantly decreased NADH, which further indi-

cated that isoflurane induced neuroprotection by up-regulation of TREK1.

In conclusion, the study demonstrates that isoflurane-induced neuroprotection in spinal cord ischemic injury is associated with TREK1. TREK1 may be a novel therapeutic target to protect the spinal cord from ischemia injury.

CONFLICT OF INTEREST

There are no conflicts of interest.

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