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 lentivirus 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; Mônaco et al., 2007). However, ischemic preconditioning is impractical for clinical practice (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 anaesthetics 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 anaesthetics including isoflurane (Gruss et al., 2004). Importantly, the activation of TREK1 mediates neuroprotection (Franks and Honoré, 2004).
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.

MATTERIAlS 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 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 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 newborn rats were disinfected with alcohol for 5-10 min and then 40 cycles 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^{-ΔΔCT} statistical method. Results were present-
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 blew 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% O$_2$, 5% CO$_2$ and 75% N$_2$ at 37°C. For the hypoxic condition group, cells were incubated in a humidified atmosphere at conditions of 93% N$_2$, 5% CO$_2$ and 2% O$_2$ 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’-CACGACCATTAATGTTAGAA-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 iso-flurane for 15 min in humidified air with 5% CO$_2$ at 37°C. The chamber was then sealed and placed inside a 37°C incubator. The concentrations of O$_2$, isoflurane and CO$_2$ 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 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 µl 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
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 ± 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...
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 neurons from severe ischemic injury, however, ischemic preconditioning is invasive for clinical practice (Dirmagl 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 (Eneydi and Czirják, 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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