Isoflurane preconditioning protects motor neurons from spinal cord ischemia: Its dose–response effects and activation of mitochondrial adenosine triphosphate–dependent potassium channel

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Received 18 May 2005; received in revised form 24 June 2005; accepted 25 June 2005

Abstract

We examined in a rabbit model of transient spinal cord ischemia (SCI) whether isoflurane (Iso) preconditioning induces ischemic tolerance to SCI in a dose–response manner, and whether this effect is dependent on mitochondrial adenosine triphosphate–dependent potassium (K_ATP) channel. Eighty-six rabbits were randomly assigned to 10 groups: Control group (n = 8) received no pretreatment. Ischemic preconditioning (IPC) group (n = 8) received 5 min of IPC 30 min before SCI. The Iso 1, Iso 2 and Iso 3 groups (n = 10, 9, 8) underwent 30 min of 1.05, 2.1 and 3.15% Iso inhalation commencing 45 min before SCI. The Iso 1HD, Iso 2HD and Iso 3HD groups (n = 9, 9, 8) each received a specific mitochondrial K_ATP channel blocker, 5-hydroxydecanoic acid (5HD, 20 mg/kg), 5 min before each respective Iso inhalation. The 5HD group (n = 8) received 5HD without Iso inhalation. The sham group (n = 9) had no SCI. SCI was produced by infra-renal aortic occlusion via the inflated balloon of a Swan–Ganz catheter for 20 min. The Iso 1, Iso 2 and Iso 3 groups showed a better neurologic outcome and more viable motor nerve cells (VMNCs) in the anterior spinal cord 72 h after reperfusion than the control group (p < 0.05). Iso 3 group showed a better neurologic outcome and more VMNCs than Iso 1 group (p < 0.05). And, the Iso 1, Iso 2 and Iso 3 groups showed a better neurologic outcome and higher VMNC numbers than the corresponding Iso 1HD, Iso 2HD and Iso 3HD groups (p < 0.05). This study demonstrates that Iso preconditioning protects the spinal cord against neuronal damage due to SCI in a dose–response manner via the activation of mitochondrial K_ATP channels.

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Paraplegia remains a devastating complication of thoracic and thoracoabdominal aortic operations. Spinal cord injury after transient ischemia can result from ischemia/reperfusion injury and apoptosis. Recently, preconditioning with volatile anesthetics such as isoflurane (Iso) has been introduced as a method of reducing ischemia/reperfusion organ injury. The exact nature of the multiple mechanisms by which Iso effects organ protection against ischemia remain unclear, but preconditioning with Iso was found to induce ischemic tolerance in heart and brain in a manner similar to ischemic preconditioning (IPC) [4,15]. In particular, mitochondrial K_ATP channel plays an important role in volatile anesthetic–induced preconditioning and in IPC [9]. The opening of mitochondrial K_ATP channels leads to the optimization of mitochondrial energy production, a decreased mitochondrial Ca2+ overload, and the increased gene expression of myocyte–inherent cytoprotective proteins.

Iso-induced preconditioning has been found to decrease myocardial infarct size after coronary artery occlusion in animal and human studies [1,5,7,12]. Also, preconditioning with Iso produced dose–dependent neuroprotection via the activation of K_ATP channel after focal cerebral ischemia in rats [15]. However, no report on the effect

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of Iso-induced preconditioning on neuroprotection against spinal cord ischemia (SCI) has been issued. This study was designed to evaluate both the dose-dependent neuroprotective effects of Iso pretreatment on ischemia/reperfusion injury in the spinal cord and the role of mitochondrial K<sub>ATP</sub> channel in the ischemic tolerance induced by Iso preconditioning in a rabbit model of transient SCI.

In this experiment, rabbits were treated in accordance with the NIH Guide for the Care and Use of Animals. All procedures and animal care protocols were approved by the Animal Care Committee of Seoul National University School of Medicine.

Eighty-six male New Zealand white rabbits weighing 2.5–3.5 kg were used in this study. The SCI models were made in the following manner: Anesthesia was induced by the intramuscular administration of ketamine (25 mg/kg) and xylazine (10 mg/kg). After tracheal intubation, lungs were mechanically ventilated with 100% oxygen. Tidal volume (15–20 ml/kg) and respiratory frequency were adjusted to maintain normocarbia. Anesthesia was maintained with intermittent xylazine (2.5 mg/kg) administered intravenously. A 5-Fr pediatric Swan–Ganz catheter (Balloon thermolidation catheter, Arrow, Reading, PA, USA) was inserted through the left femoral artery and advanced 15 cm into the abdominal aorta. Preliminary investigations confirmed that the balloon at the distal end of the catheter was positioned 0.5–1.5 cm from the left renal artery [8]. During the experiment, arterial pressures were continuously monitored both at the ear artery and the right femoral artery. Body temperature was monitored with a rectal temperature probe and maintained between 37.5 and 38.5 °C with a circulating warm water underbody heating pad.

In terms of the experimental protocol, 86 rabbits were randomly allocated to one of 10 groups. Control group (n = 8) received no pretreatment. The IPC group (n = 8) received 5 min of ischemic preconditioning 30 min before SCI. The Iso 1, Iso 2 and Iso 3 groups (n = 10, 9, 8, respectively) underwent 30 min of 1.05% (0.5 MAC), 2.1% (1.0 MAC) and 3.15% (1.5 MAC) Iso induction commencing 45 min before SCI respectively. The Iso 1HD, Iso 2HD and Iso 3HD groups (n = 9, 9, 8, respectively) each received a specific mitochondrial K<sub>ATP</sub> channel blocker, 5-hydroxydecanoic acid (5HD, 20 mg/kg), intravenously 5 min before the corresponding Iso inductions. The 5HD group (n = 8) received 5HD intravenously without Iso induction. Sham group (n = 9) received no SCI. SCI was produced by infra-renal aortic occlusion via the inflated balloon of a Swan–Ganz catheter for 20 min. This experimental model is similar to a previously described spinal cord preconditioning model [2]. After the procedure, all catheters were removed and 0.5% bupivacaine was infiltrated around the incision site to minimize pain. Rabbits recovered from anesthesia at room temperature. They were killed with a high dose of sodium pentobarbital 72 h after reperfusion, and their spinal cords were quickly removed immediately after death using the plunger of a 1 ml syringe. The tissue samples for histopathological studies were immersed in post-fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer and then stored at 4 °C. Spinal cords were cut transversely at the L4–L6 level and embedded in paraffin. Finally, they were sectioned at 4 μm.

Hind limb motor function was evaluated 3 and 72 h after reperfusion according to the Modified Tarlov Scoring (MTS) system as follows: grade 0, no movement; grade 1, slight movement or minimal anti-gravity activity; grade 2, sit with assistance or active anti-gravity activity; grade 3, sit alone; grade 4, weak hop; grade 5, normal hop [13]. Two investigators without knowledge of the treatment independently graded neurologic scores.

Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. An investigator, unaware of the animal groups and neurologic outcomes, examined each slide with a light microscope to count the numbers of viable motor nerve cells (VMNCs) in the anterior spinal cord (anterior to an imaginary line drawn through the central canal perpendicular to the vertical axis). Two additional sections cut from the lumbar spinal region (L4–L6) in each rabbit were examined to prevent bias from a single section and averages were used.

For deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining, three sections cut from the lumbar spinal region (L4–L6) in each rabbit were examined. TUNEL staining was performed to detect DNA fragmentation in cell nuclei using an ApopTag® kit (Chemicon, Temecula, CA, USA) using a modified version of the manufacturer’s instructions. In the study, if a motor nerve cell had a nucleus stained dark brown, with or without chromatin condensation, it was considered a positive cell.

All numerical data are given as the mean ± S.D. Hind limb motor function, and the numbers of VMNCs and apoptotic motor nerve cells in the anterior spinal cord were analyzed using a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney U-test. The correlation between hind limb motor function 72 h after reperfusion and VMNC numbers in ventral gray matter was tested using Pearson’s correlation coefficients. p values of <0.05 were considered statistically significant.

Total 100 rabbits were enrolled in this study, but 14 rabbits (one rabbit in the control group, four in the 5HD group, three in each of the isoflurane 1HD, isoflurane 2HD and isoflurane 3HD groups) were excluded because of survival failure.

Neurologic results are summarized in Table 1. Delayed motor dysfunction was observed in 33 of the 86 rabbits. The IPC, Iso 1, Iso 2 and Iso 3 groups had a better neurologic outcome (high MTS) 72 h after reperfusion than the control group (p < 0.05). The Iso 1 group showed a better neurologic outcome than Iso 1HD (p < 0.05), whereas Iso 3 showed a better neurologic outcome than Iso 1, Iso 1HD, Iso 2HD and Iso 3HD groups (p < 0.05 versus Iso 1, p < 0.01 versus others).

The numbers of VMNCs counted in the ventral gray matter region are shown in Table 2. VMNC numbers in the IPC, Iso
Values are mean ± S.D. Control group, spinal cord ischemia for 20 min; IPC group, ischemic preconditioning for 5 min before 20 min of SCI; Iso 1, Iso 2, Iso 3 groups, isoflurane 0.5, 1.0, 1.5 MAC inhalation for 30 min 45 min before SCI, respectively; Iso 1HD, Iso 2HD, Iso 3HD groups, administration of 5-hydroxydecanoic acid (20 mg/kg) for 5 min before isoflurane inhalation, Sham group, no SCI. Significant differences in neurologic outcome were observed between these groups 72 h after reperfusion.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cells (VMNC)</th>
<th>Number of cells (TUNEL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>53.3 ± 16.3</td>
<td>9.2 ± 11.2</td>
</tr>
<tr>
<td>Iso 1 (n=10)</td>
<td>74.6 ± 9.2 ± 5</td>
<td>0.0 ± 0.0 ± 3</td>
</tr>
<tr>
<td>Iso 2 (n=9)</td>
<td>55.6 ± 17.8 ± 1</td>
<td>0.0 ± 1.0 ± 1</td>
</tr>
<tr>
<td>Iso 3 (n=9)</td>
<td>66.8 ± 16.2 ± 1</td>
<td>0.2 ± 0.4 ± 1</td>
</tr>
<tr>
<td>Iso 1HD (n=9)</td>
<td>73.7 ± 11.3 ± 3</td>
<td>0.0 ± 0.0 ± 3</td>
</tr>
<tr>
<td>Iso 2HD (n=9)</td>
<td>100.8 ± 6.4 ± 3.1</td>
<td>0.0 ± 0.0 ± 0.0 ± 3.1</td>
</tr>
<tr>
<td>Iso 3HD (n=8)</td>
<td>36.9 ± 22.0 ± 5.5</td>
<td>3.3 ± 4.6 ± 6.6</td>
</tr>
<tr>
<td>Sham (n=9)</td>
<td>37.2 ± 15 ± 3.3</td>
<td>2.3 ± 3.3 ± 3.3</td>
</tr>
<tr>
<td>Sham (n=8)</td>
<td>42.3 ± 13 ± 5.5</td>
<td>2.0 ± 2.8 ± 8.8</td>
</tr>
<tr>
<td>Sham (n=8)</td>
<td>44.1 ± 18.9 ± 7.6</td>
<td>3.3 ± 4.8 ± 8.6</td>
</tr>
</tbody>
</table>

1. Iso 2 and Iso 3 groups were greater than in the control group (Fig. 1, p < 0.01). MTS correlated well with VMNC numbers in the ventral gray matter 72 h after reperfusion (r = 0.89, p < 0.01). VMNC numbers in the Iso 3 group were greater than in Iso 1, Iso 1HD, Iso 2HD and Iso 3HD (p < 0.05 versus Iso 1, p < 0.01 versus others).

In terms of TUNEL staining, a positive finding was observed in 27 rabbits (31%) with a low MTS (0–2) and a low VMNC number 72 h after reperfusion. Apoptotic motor nerve cells were observed in three rabbits in Iso 1 group, two in Iso 2 group, seven in the control group, five in 5HD group, four in Iso 1 HD group, three in Iso 2 HD and in Iso 3 HD, respectively (Fig. 2). In the IPC, Iso 1, Iso 2, Iso 3 and sham groups, apoptotic motor nerve cells numbers were significantly fewer than those in the control group (Table 2, p < 0.05 versus Iso 1 and Iso 2 groups, p < 0.01 versus other groups).

Recently, preconditioning with volatile anesthetics, such as Iso, has been introduced as a means of reducing organ ischemia/reperfusion injury. Our results show that Iso preconditioned groups had a better neurologic outcome and higher VMNC numbers in the anterior spinal cord than the control group. This finding suggests that preconditioning with Iso induces ischemic tolerance in the spinal cord as well as in the heart and brain. In addition, as the dose of Iso administrated was increased this effect became more obvious. Our results suggest that the effect of Iso-induced preconditioning on neuroprotection against SCI is dependent on the dose of Iso administered. This finding both confirms and expands the findings of previous reports, namely that Iso preconditioning decreases myocardial and cerebral infarct sizes following transient coronary and middle cerebral artery occlusion, respectively, in vivo, and diminishes cerebellar Purkinje cell death in vitro in a dose-dependent manner [7,15,16].

The precise natures of the multiple mechanisms by which Iso effects organ protection against ischemia remain unclear. However, the mechanism of Iso-induced preconditioning seems to have multiple complex signal transduction pathways and to share some of the mechanisms of IPC [4]. In particular, mitochondrial KATP channels play a pivotal role in volatile anesthetic-induced preconditioning and in classic IPC [4,9]. Diazoxide, a potent and specific mitochondrial KATP channel opener, reduced neurologic injury in a rabbit model of SCI and decreased myocardial infarct size after coronary artery occlusion. But, this beneficial effect was abolished by 5HD and glibenclamide (a KATP channel blocker) respectively [2,3]. In addition, the cardioprotection induced by Iso preconditioning was abolished by 5HD or glyburide (a KATP channel blocker) [4,10]. Glibenclamide also abolished the ischemic tolerance induced by Iso preconditioning in the brain [15]. These evidences indicate that mitochondrial KATP channel is a fundamental aspect of volatile anesthetic-induced preconditioning in the heart and the brain. Initially, we postulated that the intracellular signal transduction pathways of Iso preconditioning in the spinal cord might be similar to those in the heart and brain. We expected that
Fig. 1. Representative photomicrographs of spinal cord sections stained with hematoxylin and eosin. A number of viable motor neuron cells were preserved in the sham group (A), IPC group (B), ISO 1 group (C), ISO 2 group (D) and ISO 3 group (E), whereas fewer motor neuron cells and prominent vacuolization were observed in the control group (F), 5HD group (G), ISO 1HD group (H), ISO 2HD group (I) and ISO 3HD group (J, A–J; original magnification, ×100). The last section from a rabbit with paraplegia in the control group shows an ischemic red neuron (K; original magnification, ×400). The arrows indicate ischemic red neuron cells showing cytoplasmic shrinkage, nuclear condensation, and loss of Nissl body.

Apoptosis, programmed cell death, is characterized by chromatin condensation, nuclear blebbing, cellular shrinkage, and DNA fragmentation. A previous report showed that apoptosis contributes to delayed and selective motor neuron death after transient SCI [11]. Moreover, Iso also affects apoptosis. Wise-Faberowski et al. [14] reported that oxygen and glucose deprivation caused significant cerebral cortical cultured neuron apoptosis, but that pretreatment and continued treatment with halothane or Iso during the period of oxygen and glucose deprivation resulted in a concentration-dependent attenuation of this apoptosis. Iso delays, but does not prevent, the development of cerebral infarction caused by focal cerebral ischemia. Iso reduced the development of apoptosis during the early period (1 day) after ischemia, but did not prevent it during the later period, i.e., ≥4 days after ischemia [6]. Our results show that apoptotic motor nerve cell numbers in the Iso 1 and Iso 2 groups were significantly higher than in the control group 72 h after reperfusion, and no apoptotic motor nerve cells were observed in Iso 3. This result suggests that Iso pretreatment reduces, or at least delays, neuronal apoptosis.

In conclusion, our study demonstrates that Iso preconditioning protects the spinal cord against neuronal damage due to SCI in a dose–response manner via the activation of mitochondrial K<sub>ATP</sub> channel activation. Thus, as was expected, our results show that 5-HD, a specific mitochondrial K<sub>ATP</sub> channel blocker abolished the neuroprotection induced by Iso (0.5, 1 and 1.5 MAC) preconditioning when administrated before isoflurane preconditioning. This result reconfirms the validity of previous studies, which found that mitochondrial K<sub>ATP</sub> channel plays an important role in volatile anesthetic-induced preconditioning.

The neuroprotection induced by Iso preconditioning in the spinal cord would be mediated by mitochondrial K<sub>ATP</sub> channel activation. Thus, as was expected, our results show that 5-HD, a specific mitochondrial K<sub>ATP</sub> channel blocker abolished the neuroprotection induced by Iso (0.5, 1 and 1.5 MAC) preconditioning when administrated before isoflurane preconditioning.
of mitochondrial $K_{ATP}$ channels in a rabbit model of SCI.

Acknowledgment

We thank Dr. Ghee-Young Choe for his pathological advice. Supported in part by a grant number (02-04-010) from the Seoul National University Bundang Hospital research fund.

References


