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백서의 일과성 전뇌허혈 모델에서 sevoflurane 후처치 후 PKC 경로를 통한 Nrf2 와 HO-1 의 발현 증가

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의학과 마취통증의학과전공

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지도교수 박 희 평

이 논문을 의학박사 학위 논문으로 제출함

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이 한 나

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부위원장 _____ (인)

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Sevoflurane post-conditioning increases nuclear factor erythroid 2-related factor and hemoxygenase-1 expression via protein kinase C pathway in a rat model of transient global cerebral ischemia

by
Hannah Lee

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Approved by Thesis Committee:

Professor _____ **Chairman**

Professor _____ **Vice chairman**

Professor _____

Professor _____

Professor _____

ABSTRACT

Introduction: The antioxidant mechanism of sevoflurane postconditioning-induced neuroprotection remains unclear. We determined whether sevoflurane postconditioning induces nuclear factor erythroid 2-related factor (Nrf2, a master transcription factor regulating antioxidant defense genes) and heme oxygenase-1 (HO-1, an antioxidant enzyme) expression, and whether protein kinase C (PKC) is involved in Nrf2 activation in a rat model of transient global cerebral ischemia/reperfusion (IR) injury.

Methods: 86 rats were assigned to five groups: sham (n=6), control (n=20), sevoflurane postconditioning (two cycles with 2 vol% sevoflurane inhalation for 10 min, n=20), chelerythrine (a PKC inhibitor; 5 mg kg^{-1} intravenous administration, n=20), and sevoflurane postconditioning plus chelerythrine (n=20). Each group was further divided into two subgroups, based on the day of sacrifice (1 or 7 days after ischemia), of 10 rats each, except sham group (n=3). The levels of nuclear Nrf2 and cytoplasmic HO-1 were assessed.

Results: On day 1 post-ischemia, but not day 7, Nrf2 and HO-1 expression was significantly higher in the sevoflurane postconditioning group than in the control group. Chelerythrine administration reduced Nrf2 and HO-1 expression induced by sevoflurane postconditioning.

Conclusions: Sevoflurane postconditioning increased Nrf2/HO-1 expression via PKC signaling in the early phase after transient global cerebral IR injury, suggesting that activation of antioxidant enzymes may be responsible for sevoflurane postconditioning-induced neuroprotection in the early phase after

cerebral IR injury.

Keywords: Heme Oxygenase-1, GA-Binding Protein Transcription Factor, Protein Kinase C, Anesthetics, Inhalation

Student number: 2012-30519

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INTRODUCTION

The most critical pathophysiological feature of cerebral ischemia/reperfusion (I/R) injury is oxidative stress. Reactive oxygen species play a major role in oxidative stress, triggering multiple intracellular signaling pathways, including antioxidant defenses (1, 2). Although the exact mechanism is unknown, sevoflurane postconditioning seems to enhance antioxidant effects by enhancing antioxidant enzyme activities (3, 4)

Nuclear factor erythroid 2-related factor (Nrf2) is a master transcription factor regulating antioxidant defense genes (5). It appears after ischemia in the heart, kidney, liver, and brain. It binds to antioxidant response elements (AREs) in the nucleus under conditions of oxidative stress, promoting the production of antioxidant enzymes such as heme oxygenase-1 (HO-1), suggesting that Nrf2 plays an important role in protecting organs from I/R injury (1, 6-9). Recent studies showed that erythropoietin and ursolic acid (a well-known antioxidative agent and natural peptide isolated from edible plants in the Oleaceae family) provided neuroprotection by upregulating Nrf2 and HO-1 expression in a cerebral ischemia model (10, 11) Previous investigations indicated that several protein kinase C (PKC) isoforms can mediate the phosphorylation of Nrf2, leading to the nuclear accumulation of Nrf2 (1, 12). Ye et al also demonstrated that neuroprotection induced by sevoflurane postconditioning was mediated through the upregulation of HO-1 expression via a phosphoinositide-3-kinase (PI3K)/Akt pathway in rats suffering from focal cerebral I/R injuries (4).

In this study, we determined whether sevoflurane postconditioning induces Nrf2 and HO-1 expression, and whether PKC is involved in Nrf2 activation in a rat model of transient global cerebral IR injury. Furthermore, we investigated changes in the activities of Nrf2 and HO-1 over time.

MATERIALS AND METHODS

The animal protocol was in accordance with national guidelines and ARRIVE guidelines and was approved by the Seoul National University Institutional Animal Care and Use Committee (Seoul, Korea).

1. Group assignment

In total, 86 Sprague Dawley rats, weighing 320-360 g each, were used. They were assigned randomly to one of five groups (Figure 1): (1) the sham group (*n*=6) received no treatment, (2) the control group (*n*=20) underwent global transient ischemia, (3) the sevoflurane postconditioning group (*n*=20) underwent two sevoflurane inhalations after ischemia (each inhalation consisted of 10 min of 2.0 vol% sevoflurane inhalation, followed by a washout period of 10 min), (4) the chelerythrine group (*n*=20) received 5 mg kg^{-1} of chelerythrine intravenously 10 min before ischemia as described previously (13), and (5) the sevoflurane postconditioning plus chelerythrine group (*n*=20) received 5 mg kg^{-1} of chelerythrine intravenously 10 min before ischemia and two sevoflurane inhalations after ischemia.

Each group was further divided into two subgroups, based upon the day of sacrifice, with 10 rats in each except the sham group (*n*=3). The rats were sacrificed on day 1 or 7 after ischemia.

2. Surgical preparation

All rats were anaesthetized with an intraperitoneal injection of zoletil (1:1 combination of tiletamine 125 mg and zolazepam 125 mg, 20 mg kg^{-1}) and xylazine (5 mg kg^{-1}), followed by a continuous intravenous infusion of zoletil (10 mg kg^{-1} hr^{-1}) for maintenance. After tracheal intubation, mechanical ventilation was applied, delivering 60% nitrogen and a balance of oxygen. The respiratory rate and tidal volume were adjusted to maintain the PaCO2 between 4.7 and 6 kPa. The concentration of sevoflurane was monitored continuously by an anesthetic gas monitor (Datex-Ohmeda, Capnomac Ultima, Datex Instrumentarium Corp., Helsinki, Finland). The corneal and pedal reflexes and the response to tail pinch were intermittently checked during the surgical procedure to determine whether rats were at the surgical stage of anesthesia (14). Intravenous zoletil of 10 mg kg^{-1} and xylazine of 5 mg kg^{-1} were additionally administrated when such reflexes or the response to tail pinch was detected or increased heart rate or systolic blood pressure > 20% of the baseline value was observed during the operation. One of the femoral arteries was catheterized for continuous blood pressure monitoring and for measurements of arterial blood gases, plasma glucose, and hemoglobin. Anesthetic and experimental drugs were administered through the femoral vein. The temperature was monitored at two sites, rectally and pericranially, beneath the temporalis muscle, using a rectal probe and a 22-G needle thermistor (model TCAT-2 Temperature Controller; Harvard Apparatus, Holliston, USA), respectively. Body temperature was maintained at 37℃ by surface heating or cooling.

3. Transient global cerebral ischemia model

Both common carotid arteries were exposed and isolated after performing a midline incision between the neck and sternum. The right jugular vein was cannulated with a silicone catheter for blood withdrawal and reinfusion. Transient global ischemia was induced by bilateral common carotid artery occlusion and blood withdrawal with systemic hypotension, following the method of Smith et al (15). A laser Doppler monitoring system (Moor Instruments VMS-LDF2, Axminster, UK) was used for monitoring cerebral blood flow (CBF) on the ipsilateral side during the experiments. A 2-mm laser Doppler measuring sensor was placed 1-2 mm posterior and 4-5 mm lateral to the bregma on the left or right skull hemisphere, depending on the highest blood perfusion units after a small midline skin incision on the ipsilateral side. The Doppler sensor was fixed with bone cement. After heparinization (50 U), blood was quickly withdrawn through the jugular vein. When the mean arterial blood pressure (MAP) reached 26-30 mmHg and the reduction in regional CBF was more than 50% compared with baseline, both common carotid arteries were clamped with vascular clips. The MAP at 26-30 mmHg and regional CBF reduced by more than 90% compared with baseline were maintained during the ischemic period. After 10 min of ischemia, the clips were removed from both arteries and blood was slowly reinfused. After completion of the procedure, all catheters were removed and 0.5% bupivacaine was infiltrated around the incision site to relieve pain. The incision was sutured with surgical silk. The rats were placed back in their cages and allowed to recover from the anesthesia at room temperature.

Arterial blood gases, hemoglobin, and the MAP were measured 10 min before ischemia and 30 min after reperfusion. Neurological outcome was assessed by an investigator who was unaware of animal groups at 24 hour and 7 day after ischemia using modified neurological deficit score described by Katz et al (16) (Table 1).

4. Histopathological analyses

At 1 day or 7 days after ischemia, rats were anaesthetized with an intraperitoneal injection of zoletil (20 mg kg^{-1}) and then decapitated. The brains were quickly removed and divided transversely into two parts using a rat brain slice matrix. The anterior part of each brain was placed in liquid nitrogen and stored at -80℃ for Western blot analysis, while the posterior part was fixed in buffered 10% formalin for histopathological examination.

Paraffin wax-embedded brain sections were sliced into serial coronal 5 μm thick sections, and stained with hematoxylin and eosin (H&E). An investigator unaware of the group assignment evaluated necrotic and viable neurons in the hippocampal CA1 region by light microscopy. Total numbers of viable and necrotic neurons were calculated for each slide. The percentage of viable CA1 neurons was calculated as the percentage of viable neurons to total neurons. In total, six optical fields (left: 3, right: 3) were examined in the hippocampal CA1 sector under high-power magnification (×400).

To detect DNA fragmentation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using an Apoptag Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore Corp., Billerica,

USA) according to the manufacturer's protocol. TUNEL-positive neurons that contained apoptotic bodies were stained dark brown in the nucleus and were identified as apoptotic cells. The total number of apoptotic cells was counted under high-power magnification (×400) in the same manner as for H&E staining. The percentage of TUNEL-positive cells was calculated as the percentage of TUNEL-positive cells to the total cell number.

5. Western blot analysis

Western blotting for Nrf2, caspase 3, and HO-1 was performed with monoclonal anti-rabbit antibodies against Nrf2 and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, USA), and an anti-goat antibody to HO-1 (Santa Cruz Biotechnology), respectively, using a protein concentration (40 μg) shown to be within the linear range of the assay. Cytosolic protein and nuclear protein were extracted using NE-PER® nuclear and cytoplasmic extraction reagents kit (Thermo Scientific, Waltham, USA) according to the manufacturer`s instruction. Briefly, tissues from the anterior part of brain were washed with PBS (0.1M phosphate, 0.15M sodium chloride, pH 7.2) and centrifuged at 500 rpm for 5 minutes. After removal of supernatant, the leaving cell pellet was homogenized in the cytoplasmic extraction reagent (CER). An ice-cold CER II was added to the suspended cell pellet, followed by centrifugation 16000 rpm for 5 minutes. The supernatant (cytoplasmic extract) was immediately transferred to the pre-chilled tube. The insoluble pellet was suspended in ice-cold nuclear extraction reagent and centrifuged at 16000 rpm for 10 minutes in a microcentrifuge. The supernatant (nuclear

extract) was immediately transferred to the pre-chilled tube. Proteins were detected using an Amersham ECL Kit (Amersham, UK) and a densitometric analysis was used to quantify the intensities of the bands. β-actin and histone H3 intensity levels were used as control values for cytoplasmic proteins (HO-1 and caspase 3) and a nuclear protein (Nrf2) respectively (4, 17).

6. Statistical analyses

A previous study using a rat model of transient focal ischemia showed that the mean HO-1/β-actin ratio in the control group was about 48% 24 hr after ischemia and its standard deviation was about 8% (4). Sample size was calculated to have a power of 90% at α level of 0.05 to detect 12% difference in the HO-1/β-actin ratio between the control group and the sevoflurane postconditioning group.

All values are expressed as median and full range. Physiological variables, neurological deficit scores, the percentages of viable and TUNEL-positive cells in the hippocampal CA1 region, and relative protein levels of Nrf2, HO-1, and caspase-3 were compared using a repeated-measure ANOVA for time-by-group effects, followed by the Kruskal-Wallis test and Mann-Whitney U-test to compare the data at each time point. P values < 0.05 were considered to indicate statistical significance.

Group	Surgical preparation		Ischemia	Reperfusion			
Control group	Surgical preparation		Occlusion				
Chelerythrine group	Surgical preparation	Che	Occlusion				
Sevo postconditioning group	Surgical preparation		Occlusion	Sevo 2.0 %	Washout	Sevo 2.0 %	
Sevo postconditioning + chelerythrine group	Surgical preparation	Che	Occlusion	Sevo 2.0 %	Washout	Sevo 2.0 %	
Sham group	Surgical preparation						
Time	30 min	10 min	10 min	10 min	10 min	10 min	1 or 7 days

Figure 1. Experimental procotol for transient global ischemia/reperfusion injury of rats. Sevo, sevoflurane; Che, chelerythrine.

Table 1. Modified neurologic deficit scoring system

	Score
General behavioral deficit (worst = 10 points)	
Consciousness	
Attempt to explore spontaneously	0
No attempt to explore spontaneously (comatose)	5
Respiration	
Normal	0
Abnormal	5
Cranial nerve reflexes (worst = 20 points)	
Olfactory (sniffing food)	
Yes	0
No	4
Vision (follows hand)	
Yes	0
No	4
Corneal reflex	
Yes	0
No	4
Whisker movement	
Yes	0
No	4
Hearing (turns to clapped hands)	
Yes	0
No	4
Motor deficit (worst = 10 points)	
Leg/tail movement	
Normal	0
Stiff	5
Paralyzed	10
Sensory deficit (worst = 10 points)	
Leg/tail (on pinching)	
Yes	0
No	10
Coordination deficit (worst = 10 points)	
Beam balance test	
Walks the balance beam flawlessly and completes the walk within 6s	0
Walks the beam but is somewhat unsteady. Completes the walk within 6s	2 4
Walks the beam but is somewhat unsteady. May pause 1 or more times. Takes > 6s to complete the walk	6
Walks the beam, but is very unsteady, almost falling off, may pause 1 or more times, and/or takes > 6s	8
Falls off the beam before completing the walk	10
Falls off the beam immediately	
General impression (worst = 10 points)	
Normal except for the above	0
Abnormal except for the above (eg, hyperactivity and hypoactivity)	10
Total	70

RESULTS

Physiologic variables and neurological function after transient global ischemia

Of the physiological variables examined, including blood gases, heart rate, mean blood pressure, and glucose, there was no significant difference among the groups 10 min before ischemia and 30 min after ischemia (Table 2). Sevoflurane postconditioning induced 15 [7-24]% and 16 [10-21]% reduction in systolic blood pressure from baseline levels within sevoflurane postconditioning and sevoflurane postconditioning plus chelerythrine groups respectively ($P < 0.05$, respectively) whereas chelerythrine did not induce any hemodynamic changes. The CBF in all groups, except the sham group, decreased during ischemia by 90-95% from baseline, and was restored during reperfusion. Except for the sham group, there was no significant difference in CBF among the other groups. Neurological deficit scores were significantly higher in the control group than in the other groups except for the chelerythrine group at 24-h and 7-d post-ischemia ($P < 0.05$, respectively, Table 3). Sevoflurane postconditioning group had significantly lower neurological deficit scores, compared with sevoflurane plus chelerythrine group ($P < 0.05$, Table 3).

Effect of sevoflurane postconditioning on histopathologic change after transient global ischemia

In H&E sections, on days 1 and 7 after ischemia, many necrotic neurons with a shrunken cytoplasm and pyknotic or karyolytic nuclei were observed in the control group, whereas no apparent necrotic cells were detected in the sham group (Figure 2A). The median [range] percentage of necrotic cells was significantly lower in the sevoflurane postconditioning group than the control or the sevoflurane plus chelerythrine group in the hippocampal CA 1 region on day 1 (22 [17-25] vs. 36 [27-42] and 33 [27-38] ; $P < 0.01$; Figure 2B) and day 7 (44 [35-50] vs. 79 [72-84] and 54 [51-67] ; $P < 0.01$) after ischemia.

On TUNEL staining, many TUNEL-positive cells were observed in the control group, whereas no TUNEL-positive cells were detected in the sham group (Figure 3A). The median percentage of apoptotic cells was significantly lower in the sevoflurane postconditioning group than in the control and sevoflurane plus chelerythrine groups on day 1 (15[11-20] vs. 32 [20-42] and 22 [18-28] ; $P < 0.01$; Figure 3B) and day 7 (27 [23-41] vs. 65 [60-79] and 51 [40-60] ; $P < 0.01$) after ischemia.

Effect of sevoflurane postconditioning on expression of nuclear Nrf2 and cytoplasmic HO-1

Figures 4 and 5 show the results of Western blotting for Nrf2 and HO-1. The relative protein levels of Nrf2 and HO-1 were very low in the sham group. Compared with the sham group, the expression of Nrf2 and HO-1 was significantly higher in the control and all other groups on days 1 and 7 after ischemia ($P < 0.01$). The levels of Nrf2 and HO-1 were significantly higher in the sevoflurane postconditioning group than in the control group on day 1

(Nrf2: 30 [27-39] vs. 10 [8-17] %; $P < 0.01$; Figure 4B, HO-1: 18 [15-21] vs. 4 [4-9] %; $P < 0.01$; Figure 5B), but not day 7 (Nrf2: 9 [7-11] vs. 8 [6-10] %; $P > 0.05$; Figure 4B, HO-1: 4 [3-5] vs. 4 [3-5] %; $P > 0.05$; Figure 5B), after ischemia. The relative levels of Nrf2 and HO-1 were higher on day 1 than on day 7 after ischemia within the same group in all groups except the sham group ($P < 0.01$; Figure 4B; Figure 5B).

Effect of sevoflurane postconditioning on expression of caspase-3

Western blot analysis showed that caspase-3 activity was barely seen in the sham group (Figure 6A). The expression of caspase-3 was more markedly reduced in the sevoflurane postconditioning group than in the control group on days 1 (5 [4-6] vs. 10 [8-13] %; $P < 0.01$) and 7 (11 [3-15] vs. 19 [15-21] %; $P < 0.01$) after ischemia (Figure 6B).

Involvement of nuclear Nrf2 activation via a PKC pathway in sevoflurane postconditioning

Western blot analysis showed that the levels of Nrf2 and HO-1 were significantly lower in the sevoflurane postconditioning plus chelerythrine group than in the sevoflurane postconditioning group on day 1 (Nrf2: 20 [16-28] vs. 30 [27-39] %; $P < 0.01$; Figure 4B, HO-1: 10 [7-13] vs. 18 [15-21] %; $P < 0.01$; Figure 5B), but not day 7 (Nrf2: 8 [6-10] vs. 9 [7-11] %; $P > 0.05$; Figure 4B, HO-1: 4 [2-5] vs. 4 [3-5] %; $P > 0.05$; Figure 5B), after ischemia. Regarding caspase-3, the expression of caspase-3 in the sevoflurane plus chelerythrine group was higher than that in the sevoflurane postconditioning

group on day 1 (8 [7-11] vs. 5 [4-6] %; $P < 0.01$; Figure 6B) and day 7 (15 [12-16] vs. 11 [13-15] %; $P < 0.01$). The relative level of caspase-3 was higher on day 7 than on day 1 after ischemia in all groups except the sham group ($P < 0.01$).

Table 2. Physiologic variables 10 min before ischemia and 30 min after ischemia in rats.

	Sham (*n*=6)	Control (*n* =20)	Sevoflurane postconditioning (*n* =20)	Chelerythrine (*n* =20)	Sevoflurane postconditioning + chelerythrine (*n* =20)
pH					
Before	7.42 (7.36-7.46)	7.38 (7.32-7.43)	7.36 (7.32-7.40)	7.38 (7.32-7.40)	7.37 (7.30-7.42)
After	7.41 (7.38-7.44)	7.36 (7.29-7.41)	7.35 (7.25-7.38)	7.36 (7.29-7.39)	7.36 (7.30-7.38)
$PaCO_2$; kPa					
Before	4.9 (4.4-5.5)	5.3 (4.8-5.9)	5.5 (4.4-5.7)	5.1 (4.4-5.5)	5.3 (4.4-5.9)
After	4.9 (4.3-5.6)	5.6 (4.8-6.5)	5.9 (4.9-6.3)	5.5 (4.4-5.9)	5.7 (4.1-6.1)
PaO_2; kPa					
Before	28.0 (26.0-36.0)	27.8 (24.8-30.8)	29.9 (27.2-32.9)	28.0 (21.3-33.9)	29.2 (23.7-34.4)
After	29.3 (23.5-33.5)	27.7 (24.8-30.6)	28.3 (25.1-31.1)	26.3 (23.3-30.9)	27.7 (22.0-31.3)
Hemoglobin; g/dL					
Before	15 (13-16)	14 (12-15)	15 (12-16)	14 (12-16)	15 (12-16)
After	15 (12-17)	15 (13-16)	14 (11-15)	14 (12-16)	14 (13-15)
MAP; mmHg					
Before	105 (90-116)	99 (79-108)	104 (89-118)	95 (89-118)	104 (83-116)
After	97 (86-112)	102 (76-140)	95 (82-104)	101 (81-112)	94 (78-107)
Glucose; mg/dL					
Before	140 (137-173)	148 (125-164)	127(115-158)	125 (110-174)	130 (113-173)

Table 3. Modified neurological deficit score on days 1 and 7 following transient global cerebral ischemia.

Group	Modified neurological deficit score	
	Day 1	Day 7
Sham		
1 day group(*n*=3)	2 (1-3)	
7 day group (*n*=3)	2 (1-3)	2 (1-3)
Control		
1 day group (*n*=10)	10 (6-14)*	
7 day group (*n*=10)	9.5 (6-16)*	10 (7-17)*
Sevoflurane postconditioning		
1 day group (*n*=10)	6 (4-8)	
7 day group (*n*=10)	6 (4-9)	6 (4-8)
Chelerythrine		
1 day group (*n*=10)	9 (7-14)	
7 day group (*n*=10)	9 (7-15)	9.5 (8-16)
Sevoflurane postconditioning + chelerythrine		
1 day group (*n*=10)	7.5 (6-11)†	
7 day group (*n*=10)	8 (5-12)†	8 (6-11)†

Values are expressed as median (range).

*$P < 0.05$ vs. sevoflurane postconditioning and sevoflurane postconditioning + chelerythrine groups respectively. †$P < 0.05$ vs. sevoflurane postconditioning group.

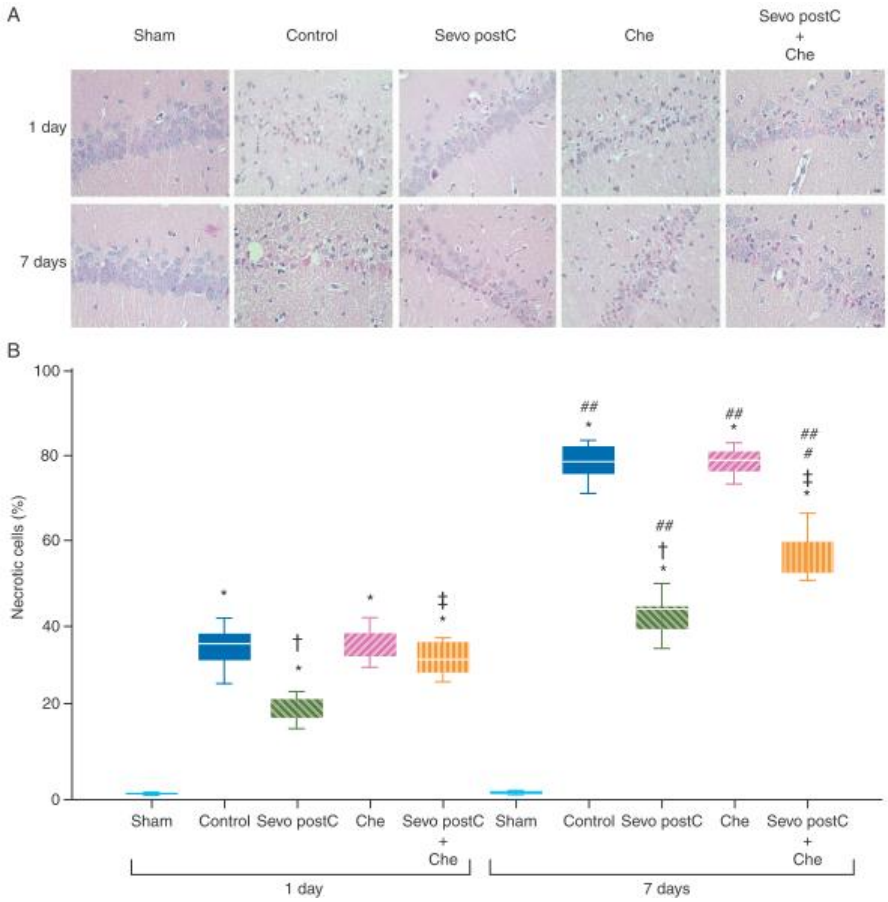

Figure 2. H&E staining of necrotic and viable cells in the hippocampal CA1 regions on days 1 and 7 following transient global cerebral ischemia.

(A) Representative photomicrographs showing necrotic and viable cells in the hippocampal CA1 regions on days 1 and 7 following transient global cerebral ischemia. **(B)** Percentages of necrotic cells in the hippocampal CA1 region in the sham, control, sevoflurane postconditioning, chelerythrine, and sevoflurane postconditioning + chelerythrine groups on days 1 and 7 following ischemia (H&E staining). The results are given as median, interquartile and full ranges. **P* < 0.01 vs. the sham group. †*P* < 0.01 vs. the control and chelerythrine groups. ‡*P* < 0.01 vs. the sevoflurane postconditioning group. #*P* < 0.05 vs. the control and chelerythrine groups. ##*P* < 0.01 vs. day 1. H&E, hematoxylin and eosin; CA1, Cornu Ammonis area 1; Sevo, sevoflurane; postC, postconditioning; Che, chelerythrine.

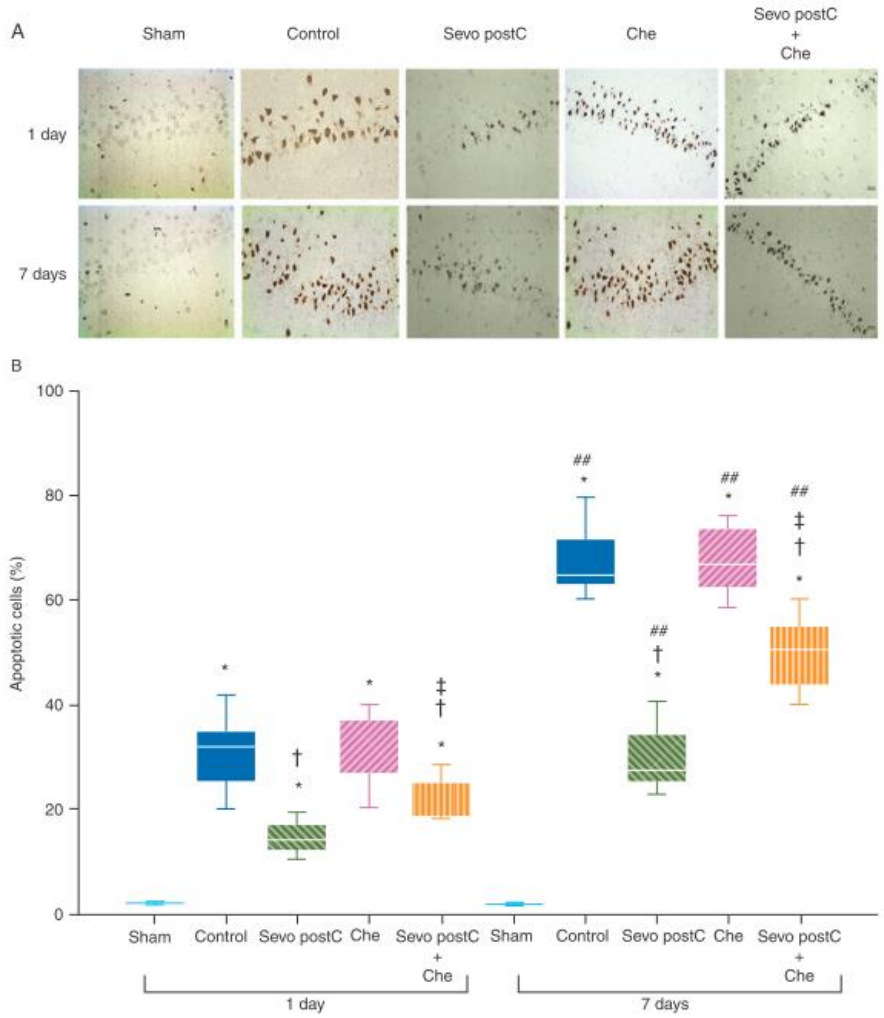

Figure 3. TUNEL staining of apoptotic cells in the hippocampal CA1 regions on days 1 and 7 following transient global cerebral ischemia

(A) Representative photomicrographs of TUNEL staining showing apoptotic cells in the hippocampal CA1 region on days 1 and 7 following transient global cerebral ischemia. **(B)** Percentages of apoptotic cells in the hippocampal CA1 region in the sham, control, sevoflurane postconditioning, chelerythrine, and sevoflurane postconditioning + chelerythrine groups on days 1 and 7 following ischemia (TUNEL staining). The results are given as median, interquartile and full ranges. **P* < 0.01 vs. the sham group. †*P* < 0.01 vs. the control and chelerythrine groups. ‡*P* < 0.01 vs. the sevoflurane postconditioning group. #P < 0.01 vs. the control and chelerythrine groups. ##*P* < 0.01 vs. day 1. TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-uracil triphosphate biotin in situ nick end labeling; CA1, Cornu Ammonis area 1; Sevo, sevoflurane; postC, postconditioning; Che, chelerythrine.

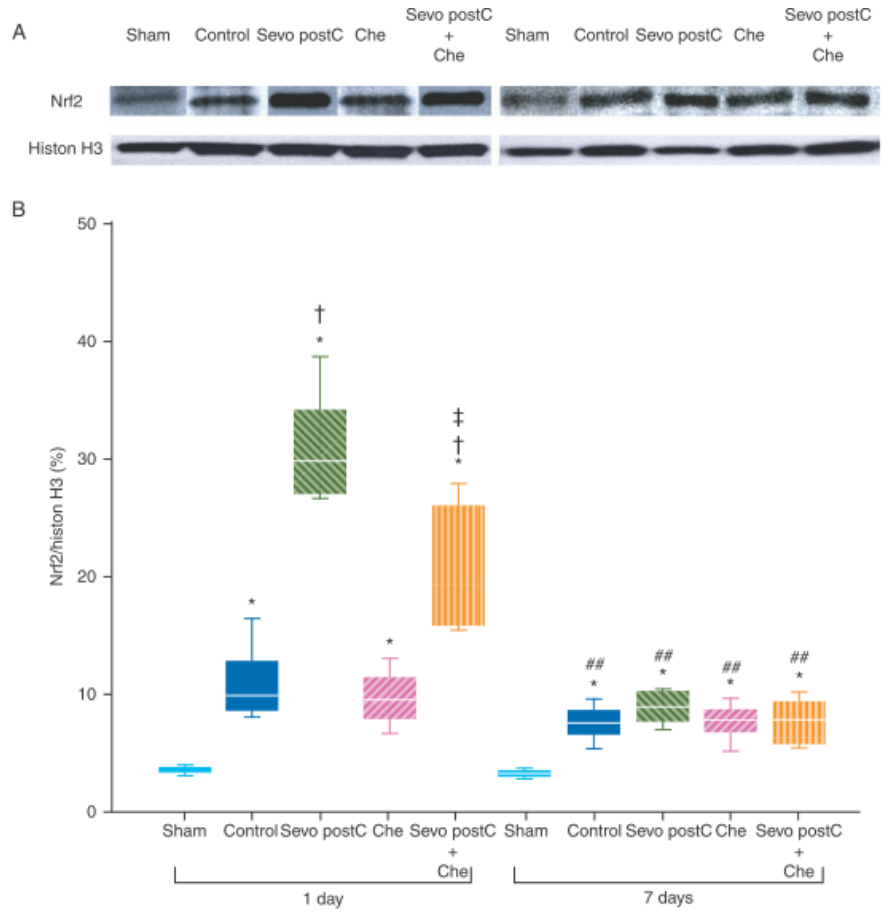

Figure 4. Western blot analysis of nuclear Nrf2 on days 1 and 7 following transient global cerebral ischemia

(A) Representative protein bands from a Western blot analysis of nuclear Nrf2 on days 1 and 7 following transient global cerebral ischemia in rats. **(B)** Densitometric evaluation of nuclear Nrf2 in the sham, control, sevoflurane postconditioning, chelerythrine, and sevoflurane postconditioning + chelerythrine groups. The results are given as median, interquartile and full ranges. Histone H3 was used as a control. **P* < 0.01 vs. the sham group. †*P* < 0.01 vs. the control and chelerythrine groups. ‡P < 0.01 vs. the sevoflurane postconditioning group. ##*P* < 0.01 vs. day 1. Nrf2, nuclear factor erythroid 2-related factor 2; Sevo, sevoflurane; postC, postconditioning; Che, chelerythrine.

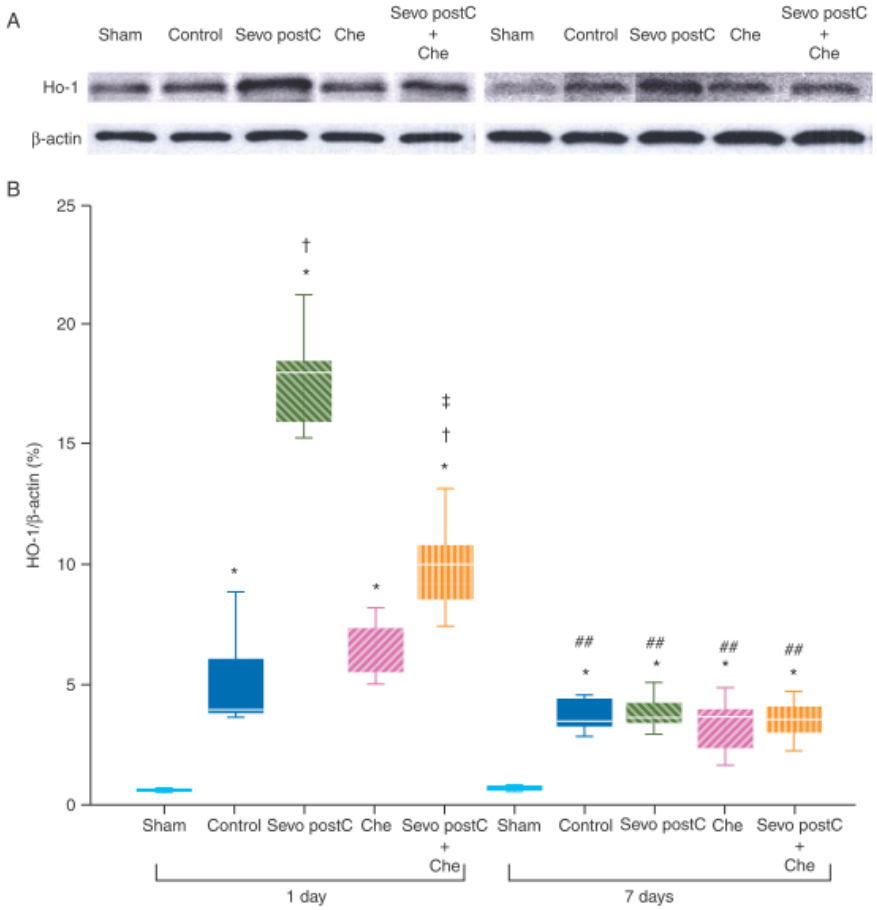

Figure 5. Western blot analysis of HO-1 on days 1 and 7 following transient global cerebral ischemia

(A) Representative protein bands from a Western blot analysis of HO-1 on days 1 and 7 following transient global cerebral ischemia. **(B)** Densitometric evaluation of HO-1 in the sham, control, sevoflurane postconditioning, chelerythrine, and sevoflurane postconditioning + chelerythrine groups. The results are given as median, interquartile and full ranges. β-actin was used as a control. *P < 0.01 vs. the sham group. †P < 0.01 vs. the control and chelerythrine groups. ‡P < 0.01 vs. the sevoflurane postconditioning group. ##P < 0.01 vs. day 1. HO-1, heme oxygenase-1; Sevo, sevoflurane; postC, postconditioning; Che, chelerythrine.

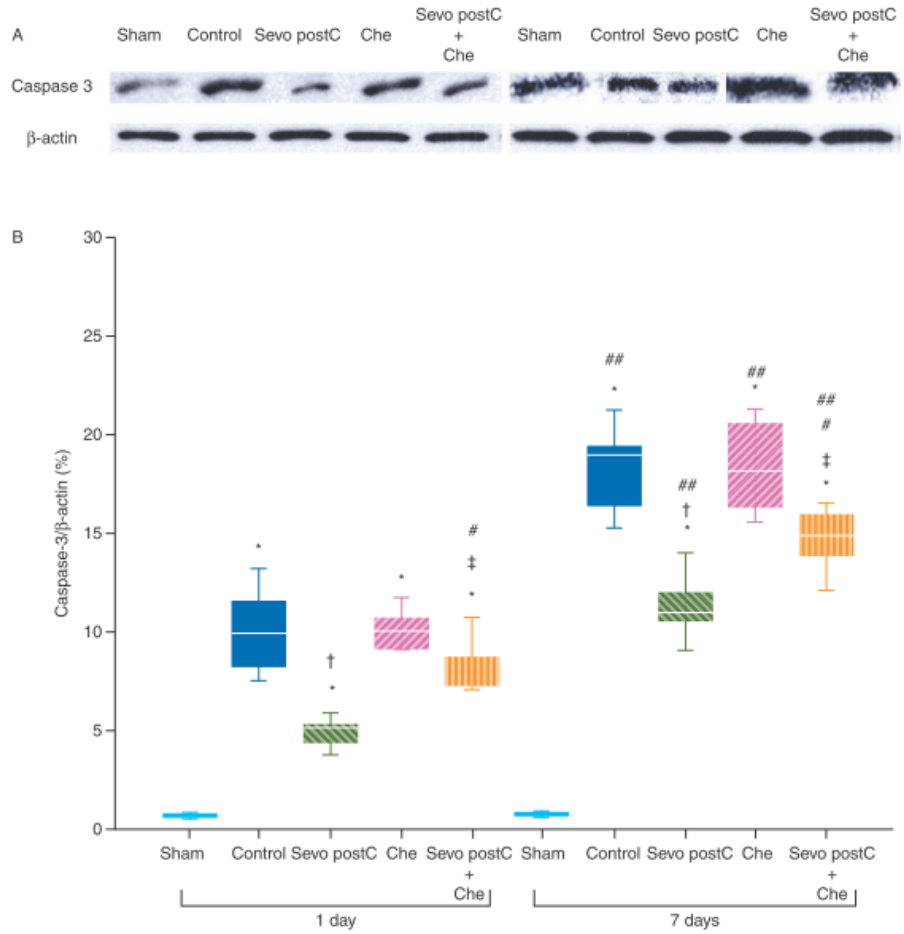

Figure 6. Western blot analysis of caspase 3 on days 1 and 7 after transient global cerebral ischemia

(A) Representative protein bands from a Western blot analysis of caspase 3 on days 1 and 7 after transient global cerebral ischemia. **(B)** Densitometric evaluation of caspase-3 in the sham, control, sevoflurane postconditioning, chelerythrine, and sevoflurane postconditioning + chelerythrine groups. The results are given as median, interquartile and full ranges. β-actin was used as a control. *P < 0.01 vs. the sham group. †P < 0.01 vs. the control and chelerythrine groups. ‡P < 0.01 vs. the sevoflurane postconditioning group. #P < 0.05 vs. the control group. ##P < 0.01 vs. day 1. Sevo, sevoflurane; postC, postconditioning; Che, chelerythrine.

DISCUSSION

In this study, we demonstrated that sevoflurane postconditioning (1) increased the expression of Nrf2 and HO-1 via a PKC pathway and (2) decreased apoptosis by attenuating the activation of caspase-3 in a rat model of transient global cerebral I/R injury.

PKC has been known as a possible target for the effects of volatile anesthetics through involvement of multiple physiological processes such as neurotransmitter release, ion channel activity, and neurotransmitter receptor desensitization (18). Among the PKC isoforms, conventional PKC isoforms are activated by lipid second messengers, the diacylglycerol (DAG) and cofactoer, Ca^{2+}, while novel PKC isoforms are activated by only DAG or phorbol esters. The activated PKCs are translocated from cytosol to cell membranes and then involved in the expression of their effects (19). Recent study suggested that sevoflurane induced translocation of PKC in mice brain (20).

PKC is also known to play a role in the nuclear translocation of Nrf2, by mediating its phosphorylation in response to oxidative stress, leading to the activation of AREs and subsequent antioxidant enzyme production (1, 12). Consistent with our results, there is experimental evidence supporting an association between Nrf2 activation and the PKC pathway. In an in vitro model of neurodegenerative disease, activation of a PKC signaling pathway regulated Nrf2 (21). Quesada et al also suggested that activation of a PKC/Nrf2 pathway was a potential mechanism for the neuroprotection of C-

terminal mechano-growth factor, a variant of insulin-like growth factor-1(22) In an isolated rabbit heart model of ischemic preconditioning, Zhang et al reported that PKC participates in the activation of Nrf2 signaling (17)

Nrf2 is a master redox regulator of antioxidant defense genes, including NAD(P)H:quinone oxidoreductase-1 (NQO1), glutathione S-transferase, and HO-1 (5). Under normal conditions, Nrf2 is bound to kelch-like ECH-associated protein 1 (KEAP-1) as a complex. Antioxidant activities through the Nrf2 pathway are induced by conditions of oxidative stress, and the Nrf2/KEAP-1 complex becomes disrupted and Nrf2 is translocated to the nucleus where it binds to ARE sequences (5). Plant extracts, including luteolin and ursolic acid, showed neuroprotective effects by upregulating Nrf2 after cerebral ischemia in a focal ischemic animal model (11, 23) Ginkgo biloba, another plant extract, also provided neuroprotection via the Nrf2/HO-1 pathway in a transient global cerebral ischemia model (24). In addition, ischemic preconditioning has been shown to have neuroprotective effects against cerebral I/R injury, also by activating Nrf2 (25). In the present study, sevoflurane postconditioning provided neuroprotection by increasing Nrf2 and HO-1 expression. Similar to our result, a recent study showed that increased HO-1 expression via a PI3K/Akt pathway was involved in sevoflurane postconditioning-induced neuroprotection against focal cerebral I/R injury (4). Such findings suggest that Nrf2-mediated antioxidant enzyme production is a major neuroprotective mechanism after cerebral I/R injury.

In this study, the sevoflurane postconditioning plus PKC inhibitor group still showed a neuroprotective effect compared with the control group. This

finding suggests that in addition to the PKC pathway, other mechanism(s) may be involved in sevoflurane postconditioning-induced neuroprotection. A literature review indicated that direct activation of mitochondrial KATP channels, p38 mitogen-activated protein kinase activation, upregulation of hypoxia inducible factor-1α and HO-1 through a PI3K/Akt pathway, and inhibition of proinflammatory cytokine production have been suggested to be involved in sevoflurane postconditioning-induced neuroprotection after cerebral I/R injury (4, 26-31). Sevoflurane-induced anti-excitotoxic properties also have neuroprotective effects by reducing extracellular glutamate levels (32) and enhancing the activity of glutamate transporters (34). Recently, activation of the two-pore domain K+ channel TREK1 (33), inhibition of mitochondrial permeability transition pore opening (34), and upregulation of insulin-like growth factor-1 via Akt/c-Jun N-terminal kinase signaling pathways (35) were added to the list of mechanisms potentially involved in sevoflurane-induced neuroprotection. In this study, Nrf2 expression was still increased even after pretreatment with chelerythrine. This finding also suggests that Nrf2 can be activated by other signaling pathways than PKC mediated phosphorylation, as indicated by previous studies, in which a number of proteins such as the glycogen synthase kinase 3β, the cyclin-dependent protein kinase p21, and mitogen-activated protein kinase are involved in the regulation of Nrf2 (36-38). Additionally, the possibility that PKC is incompletely blocked by low dose of chelerythrine might be considered. But we think that the possibility is less likely to happen because

relative high dose of chelerythrine was used in this study. The dose was used in previous studies (13, 39).

The inhibition of apoptosis is a major mechanism in sevoflurane postconditioning-induced neuroprotection (17). The anti-apoptotic effects of sevoflurane may be explained by the upregulation of anti-apoptotic proteins and attenuation of proapoptotic activities. Nrf2 is involved in the increased expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL, as well as decreased caspase-3 activity (40-42). A previous study in a neurodegenerative disease model showed that attenuation of apoptotic death occurred with Nrf2 pathway modulation (43). Also, in a traumatic brain injury model, the apoptotic index was increased in Nrf2-deletion mice (44). In the present study, we also found that transient global cerebral I/R injury significantly increased apoptotic cells in the hippocampal CA1 region and enhanced caspase-3 expression, whereas sevoflurane postconditioning reduced apoptosis and attenuated the expression of caspase-3, which is an important final effector in caspase-dependent pathways of apoptosis. Such findings are supported by the results of recent studies, in which apoptosis was reduced significantly by sevoflurane postconditioning in a rat model of transient global cerebral I/R injury (26, 28). A PKC inhibitor, chelerythrine, reduced the anti-apoptotic effect of sevoflurane postconditioning in this study. Also consistent with our findings, a previous study demonstrated that electroacupuncture pretreatment reduced apoptosis in rats after transient focal cerebral I/R injury, while the neuroprotective effect was abolished by a specific epsilon PKC inhibitor (45).

Other studies have shown that the anti-apoptotic effect of ischemic preconditioning on myocardium was lost with PKC inhibition (46, 47).

In this study, we also demonstrated the time course of expression of Nrf2, HO-1, and caspase-3 at the protein level. A recent study in a rat model of focal cerebral ischemia showed that sulforaphane, an Nrf2 inducer, increased the expression of Nrf2 at 24 h after reperfusion and decreased it at 72 h after reperfusion (48). Similar to their study, the expression of Nrf2 and its target antioxidant enzyme, HO-1, increased 24 h after ischemia and declined 7 days after ischemia in this study. The levels of Nrf2 and HO-1 on day 7 post-ischemia were not significantly different between the control and sevoflurane postconditioning groups in our study. Shokeir et al reported that an apoptotic marker, caspase-3, continued to increase up to 48 h post- ischemia. In this study, the level of caspase-3 was higher 7 days after ischemia than at 1 day after ischemia (49). Taken together, an antioxidant pathway may be involved in the neuroprotection of sevoflurane postconditioning in the early phase after cerebral I/R injury, whereas an anti-apoptotic process may be involved in the delayed phase after ischemia.

Chelerythrine is a potent but nonspecific inhibitor of protein kinase C. In this study, chelerythrine was intravenously administrated to inhibit PKC activity before cerebral ischemia. Cerebral I/R injury itself can activate a certain types of PKC isoforms. Indeed, previous studies demonstrated that PKC isozymes, especially PKCδ and PKCγ, was activated by cerebral I/R injury (13, 50) A recent study in a focal cerebral ischemia model showed that mitochondrial KATP channels and PKCϵ were involved in sevoflurane preconditioning-

induced neuroprotection (51). Another report showed that inhibition of PKCδ was involved in limb remote postconditioning-induced neuroprotection (52). Chelerythrine pretreatment can inhibit PKC activation by cerebral I/R insults and would modulate not only effects of sevoflurane on PKC activity but also physiological responses to cerebral I/R injury. A previous study suggested that when nonspecific PKC inhibitors are used, experimental results should be interpreted with caution as they conceal the role of individual PKC isozymes, affecting inconsistency in reports on PKC function (53).

This study has several limitations. First, Western blotting of PKC or PKC isoforms was not performed in this study. Thus, we did not specify the PKC subtype involved in the PKC-Nrf2 pathway. Also, PKC involvement to the anti-oxidative and neuroprotective effects of sevoflurane postconditioning was not fully elucidated in this study, although some studies showed that the PKC signaling pathway was engaged in sevoflurane preconditioning-induced neuroprotection or cardioprotection (2, 48, 54). Second, the extent of Nrf2 translocation from the cytosol to nucleus was not completely investigated in this study because cytoplasmic Nrf2 level was not measured. However, previous experimental studies showed that activated nuclear Nrf2, not inactivated cytoplasmic Nrf2, played a pivot role in inducing HO-1 activation (10, 11, 48). A recent study revealed that sevoflurane postconditioning increased the Nrf2 ratio (nuclear Nrf2/ cytoplasmic Nrf2) and the messenger RNA level of HO-1 after I/R injury in a rat heart model (55). Third, we measured Nrf2, HO-1, and caspase-3 levels 1 and 7 days after ischemia. Thus, we do not know the peak levels of those proteins or the time to peak

expression. However, previous studies have demonstrated that the time to peak expression of antioxidant enzymes was 24 h after ischemia and that apoptosis increased over time (48, 49). Finally, we measured only the level of HO-1 among Nrf2-dependent antioxidant enzymes. Other Nrf2-dependent antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, NQO1, and thioredoxin-1, were not investigated.

Conclusion

In this study, we demonstrated that sevoflurane postconditioning not only reduced apoptosis but also increased Nrf2 and HO-1 expression, which was in part blocked by chelerythrine, in the early phase after transient global cerebral ischemia in a rat. Such results suggest that the antioxidant effect of sevoflurane postconditioning may be responsible for neuroprotection in the early phase after cerebral I/R injury.

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국문 초록

서론: sevoflurane 후처치로 유도된 신경보호의 항산화 기전은 명확하지 않다. 본 연구의 목적은 백서의 전뇌허혈 모델에서 sevoflurane 후처치의 뇌보호 효과가 nuclear factor erythroid 2-related factor (Nrf2) 와 Nrf2 의존성 효소들, 즉 항산화 단백질 효소들의 발현 증가를 유도시키고 세포자멸사를 감소시킴으로써 뇌보호 효과를 나타내는 지 알아보는 것이다. 또한 protein kinase C (PKC) 가 Nrf2 의존성 pathway 에 관여하는지 알아보고자 하였다.

방법: 86 마리의 쥐를 치료 약제에 따라 5 개의 군으로 나누었다: sham 군(n=6), 대조군(n=20), sevoflurane 후처치군(10 분 동안 뇌허혈 후 45 분 동안 10 분씩 2 회의 sevoflurane 2.0 vol% 흡입, n=20), chelerythrine(PKC 억제제; 전뇌허혈 10 분전 5 mg kg−1 정맥 내 투여, n=20), sevoflurane 후처치 + chelerythrine (n=20). Sham 군을 제외한 쥐에서 전뇌허혈 후 1 과 7 일에 핵 내 Nrf2 와 세포질 내 HO−1 농도를 검사하였다.

결과: 전뇌허혈 1 일 후 측정한 Nrf2 와 HO−1 농도가 sevoflurane 후처치군에서 대조군에 비해 유의하게 증가하였지만, 7 일 후 측정한 Nrf2 와 HO−1 의 농도는 대조군에 비해 유의한 변화가 없었다.

Chelerythrine 투여는 sevoflurane 후처치로 인해 증가된 Nrf2 와 NO-1 의 발현을 감소시켰다.

결론: sevoflurane 후처치는 전뇌허혈 손상시 초기에 PKC 경로를 통해 Nrf2/HO-1 발현을 증가시킨다. 이것은 sevoflurane 후처치로 인한 뇌보호 효과가 뇌허혈 재관류 손상 초기에 항산화 효소의 활성화에 의한 것임을 보여준다.

주요어 : Heme Oxygenase-1; GA-Binding Protein Transcription Factor; Protein Kinase C; 흡입마취제
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