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

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Editor’s key points

- Sevoflurane is neuroprotective, but mechanisms are unclear.
- Rats underwent cerebral ischaemia and reperfusion with and without sevoflurane and a protein kinase C (PKC) inhibitor.
- Sevoflurane increased expression of Nrf2, a key ‘master regulator’ of antioxidants, and haemoxygenase, an antioxidant enzyme, and inhibition of PKC prevented this.
- Sevoflurane is neuroprotective via PKC-induced antioxidant mechanisms.

Background. The antioxidant mechanism of sevoflurane post-conditioning-induced neuroprotection remains unclear. We determined whether sevoflurane post-conditioning induces nuclear factor erythroid 2-related factor (Nrf2, a master transcription factor regulating antioxidant defence genes) and haemoxygenase-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 ischaemia/reperfusion (I/R) injury.

Methods. Eighty-six rats were assigned to five groups: sham (n=6), control (n=20), sevoflurane post-conditioning (two cycles with 2 vol% sevoflurane inhalation for 10 min, n=20), chelerythrine (a PKC inhibitor; 5 mg kg⁻¹ i.v. administration, n=20), and sevoflurane post-conditioning plus chelerythrine (n=20). The levels of nuclear Nrf2 and cytoplasmic HO-1 were assessed 1 or 7 days after ischaemia (n=10 each, apart from the sham group, n=3).

Results. On day 1 but not day 7 post-ischaemia, Nrf2 and HO-1 expression were significantly higher in the sevoflurane post-conditioning group than in the control group. Chelerythrine administration reduced the elevated Nrf2 and HO-1 expression induced by sevoflurane post-conditioning.

Conclusions. Sevoflurane post-conditioning increased Nrf2/HO-1 expression via PKC signalling in the early phase after transient global cerebral I/R injury, suggesting that activation of antioxidant enzymes may be responsible for sevoflurane post-conditioning-induced neuroprotection in the early phase after cerebral I/R injury.

Keywords: anaesthetics, inhalation; GA-binding protein transcription factor; haeme oxygenase-1; protein kinase C

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The most critical pathophysiological feature of cerebral ischaemia/reperfusion (I/R) injury is oxidative stress, triggering multiple intracellular signalling pathways, including antioxidant defences.1,2 Although the exact mechanism is unknown, sevoflurane post-conditioning seems to augment antioxidant effects by enhancing antioxidant enzyme activities.3,4

Nuclear factor erythroid 2-related factor (Nrf2) is a master transcription factor regulating antioxidant defence genes,5 and is detectable after ischaemia in the heart, kidney, liver, and brain. Under conditions of oxidative stress, it binds to antioxidant response elements (AREs) in the nucleus, promoting the production of antioxidant enzymes such as haemoxygenase-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 anti-oxidative agent and natural peptide isolated from edible plants in the Oleaceae family) provided neuroprotection by up-regulating Nrf2 and HO-1 expression in a cerebral ischaemia model.10,11 Previous
investigations indicated that several protein kinase C (PKC) iso-
forms can mediate the phosphorylation of Nrf2, leading to
nuclear accumulation.\textsuperscript{11} Ye and colleagues\textsuperscript{4} also demonstrated
that neuroprotection induced by sevoflurane post-conditioning
was mediated via up-regulation of HO-1 expression via a
phosphoinositide-3-kinase (PI3 K)/Akt pathway in rats suffering
from focal cerebral I/R injuries.

In this study, we determined whether sevoflurane post-
conditioning 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.

\section*{Methods}

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

\subsection*{Group assignment}

In total, 86 Sprague–Dawley rats, weighing 320–360 g each,
were used. They were assigned randomly to one of the five
groups (Fig. 1): (i) the sham group (n=6) received no treatment,
(ii) the control group (n=20) underwent global transient is-
chaemia, (iii) the sevoflurane post-conditioning group (n=20)
underwent two periods of inhalation of 2\% vol. sevoflurane
10 min, followed by a washout period of 10 min, after ischae-
mia (iv) the chelerythrine group (n=20) received 5 mg kg\textsuperscript{-1}
of chelerythrine i.v. 10 min before ischaemia as described
previously,\textsuperscript{13} and (v) the sevoflurane post-conditioning plus
chelerythrine group (n=20) received 5 mg kg\textsuperscript{-1} of chelerythr-
ine i.v. 10 min before ischaemia and two periods of sevoflurane
inhalations after ischaemia. Rats were studied 1 or 7 days post-
ischaemia or sham with 10 rats in each group except the sham
group (n=3).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
 & Surgical preparation & Ischaemia & Reperfusion \\
\hline
\textbf{Control group} & Surgical preparation & Occlusion & & \\
\hline
\textbf{Chelerythrine} & Surgical preparation & Che & Occlusion & \\
\textbf{group} & & & & \\
\hline
\textbf{Sevo postconditioning} & Surgical preparation & Occlusion & Sevo & Washout & Sevo \\
\textbf{group} & & & 2.0\% & 2.0\% & 2.0\% \\
\hline
\textbf{Sevo postconditioning} & Surgical preparation & Che & Occlusion & Sevo & Washout & Sevo \\
\textbf{+ chelerythrine} & & & 2.0\% & 2.0\% & 2.0\% \\
\textbf{group} & & & & & & \\
\hline
\textbf{Sham group} & Surgical preparation & & & & & \\
\hline
\hline
30 min & 10 min & 10 min & 10 min & 10 min & 10 min & 1 or 7 days \\
\hline
\end{tabular}
\caption{Experiment protocol. Sevo, sevoflurane.}
\end{table}

\subsection*{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\textsuperscript{-1}) and xylazine (5 mg kg\textsuperscript{-1}), followed by a
continuous i.v. infusion of zoletil (10 mg kg\textsuperscript{-1} h\textsuperscript{-1}) for mainte-
rance. After tracheal intubation, rats were ventilated with 30%
or oxygen/60\% nitrogen. The respiratory rate and tidal volume
were adjusted to maintain the \textit{P}aCO\textsubscript{2} between 4.7 and 6 kPa.

The concentration of sevoflurane was monitored continuously
by an anaesthetic gas monitor (Datex-Ohmeda, Capnomac
Ultima, Datex Instrumentarium Corp., Helsinki, Finland). The
conical and pedal reflexes and response to tail pinch were
intermittently checked during the surgical procedure to ascer-
tain an appropriate level of anaesthesia.\textsuperscript{14} I.V. zoletil (10 mg
kg\textsuperscript{-1}) and xylazine (5 mg kg\textsuperscript{-1}) were additionally administered
when such reflexes or the response to tail pinch was detected
or increased heart rate or systolic arterial pressure >20\% of the
baseline value was observed during the operation. One of the
femoral arteries was catheterized for continuous arterial pres-
sure monitoring and for measurements of arterial blood gases,
plasma glucose, and haemoglobin. Anaesthetic and experi-
mental 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 Tempara-
ture Controller; Harvard Apparatus, Holliston, MA, USA),
respectively. Body temperature was maintained at 37\degree C by
surface heating or cooling.

\subsection*{Transient global cerebral ischaemia 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 ischaemia
was induced by bilateral common carotid artery occlusion and
blood withdrawal with systemic hypotension, after the method of Smith and colleagues.\(^1\)\(^5\) 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 units), blood was quickly withdrawn through the jugular vein. When the mean arterial pressure (MAP) reached 26–30 mm Hg 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 mm Hg and regional CBF reduced by more than 90% compared with baseline were maintained during the ischaemic period. After 10 min of ischaemia, 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 anaesthesia at room temperature. Arterial blood gases, haemoglobin, and their cages before ischaemia and 30 min after reperfusion. Neurological outcome was assessed by an investigator who was unaware of animal groups at 24 h and 7 days after ischaemia using modified neurological deficit score described by Katz and colleagues.\(^1\)\(^6\) The modified neurological deficit scoring system (total score = 70 points) consists of six main exams: (i) general behavioural deficit (worst = 10 points) including consciousness (0: attempt to explore spontaneously, 5: no attempt to explore spontaneously) and respiration (0: normal, 5: abnormal); (ii) cranial nerve reflexes (worst = 20 points) including olfactory (0: yes, 5: no), vision (0: yes, 5: no), corneal reflex (0: yes, 5: no), whisker movement (0: yes, 5: no), and hearing (0: yes, 5: no); (iii) motor deficit (worst = 10 points) using leg/tail movement (0: normal, 5: stiff, 10: paralysed); (iv) sensory deficit (worst = 10 points) using leg/tail movement on pinching (0: yes, 10: no); (v) coordination deficit (worst = 10 points) using beam balance test (0: walks the balance beam flawlessly and completes the walk within 6 s, 2: walks the beam but is somewhat unsteady, completes the walk within 6 s, 4: walks the beam but is somewhat unsteady, may pause 1 or more times or takes >6 s to complete the walk, 6: walks the beam, but is very unsteady, almost falling off, may pause 1 or more times, and/or takes >6 s, 8: falls off the beam before completing the walk, 10: falls off the beam immediately); (vi) general impression (worst = 10 points) such as activity (0: normal except for the above, 10: abnormal except for the above, for example, hyperactivity or hypoactivity).

**Histopathological analyses**

At 1 or 7 days after ischaemia, 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°C 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 haematoxylin and eosin (H&E). An investigator unaware of the group assignment evaluated necrotic and viable neurones in the hippocampal CA1 region by light microscopy. In total, six optical fields (left: three, right: three) were examined in the hippocampal CA1 sector under high-power magnification (×400).

To detect DNA fragmentation, terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining was performed using an Apoptag Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore Corp., Billerica, MA, USA) according to the manufacturer’s protocol. TUNEL-positive neurones 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) and the percentage of TUNEL-positive cells was calculated as the percentage of TUNEL-positive cells to the total cell number.

**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, CA, 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, MA, USA) according to the manufacturer’s instructions. Proteins were detected using an Amersham enhanced chemiluminescence kit (Amersham, UK) and densitometric analysis was used for quantification. β-Actin and histone H3 intensity levels were used as control values for cytoplasmic proteins (HO-1 and caspase 3) and nuclear protein (Nrf2), respectively.\(^5\)\(^\text{17}\)

**Statistical analyses**

A previous study using a rat model of transient focal ischaemia showed that the mean HO-1/β-actin ratio in the control group was about 48% 24 h after ischaemia 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 post-conditioning 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 analysis of variance 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 of <0.05 were considered as statistically significant.

Results

Physiological variables and neurological function after transient global ischaemia

Blood gases, heart rate, MAP, and glucose were not significantly different between groups 10 min before and 30 min after ischaemia (Supplementary Table S1). Sevoflurane post-conditioning induced reductions in systolic arterial pressure from baseline levels of 15 (7–24)% and 16 (10–21)% in the sevoflurane post-conditioning and sevoflurane plus chelerythrine groups, respectively (P<0.05, respectively), whereas chelerythrine alone did not induce any haemodynamic changes. The CBF in all groups except the sham group decreased during ischaemia 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 days post-ischaemia (P<0.05, respectively, Table 1). The sevoflurane post-conditioning group had significantly lower neurological deficit scores, compared with the sevoflurane plus chelerythrine group (P<0.05, Table 1).

Histopathological change after transient global ischaemia

On days 1 and 7 after ischaemia, many necrotic neurones 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 (Fig. 2a). The median (range) percentage of necrotic cells was significantly lower in the sevoflurane post-conditioning group than the control or the sevoflurane plus chelerythrine group in the hippocampal CA 1 region on day 1 (P<0.01) and day 7 (P<0.01) after ischaemia (Fig. 2b).

Many TUNEL-positive cells were observed in the control group, whereas no TUNEL-positive cells were detected in the sham group (Fig. 3a). The median percentage of apoptotic cells was significantly lower in the sevoflurane post-conditioning group than in the control and sevoflurane plus chelerythrine groups on day 1 (P<0.01) and day 7 (P<0.01) after ischaemia (Fig. 3b).

Expression of nuclear Nrf2 and cytoplasmic HO-1

The relative protein expression of Nrf2 and HO-1 were very low in the sham group and were significantly higher in the control and all other groups on days 1 and 7 after ischaemia (P<0.01, Figs 4 and 5). Nrf2 and HO-1 expression were significantly higher in the sevoflurane post-conditioning group than in the control group on day 1 (both P<0.01), but not day 7 after ischaemia (Figs 4b and 5a). Also Nrf2 and HO-1 expression was significantly lower in the sevoflurane plus chelerythrine group than in the sevoflurane alone group on day 1 (both P<0.01) but not day 7 after ischaemia (Figs 4a and 5a). The relative levels of Nrf2 and HO-1 were also higher on day 1 than day 7 after ischaemia in every group except the sham group (P<0.01, Figs 4b and 5a).

Expression of caspase-3

Caspase-3 expression was barely detectable in the sham group (Fig. 6a) and was lower in the sevoflurane post-conditioning group than the control group on both days 1 and 7 after ischaemia (both P<0.01, Fig. 6a). In addition, caspase-3 in the sevoflurane plus chelerythrine group was higher than that in the sevoflurane alone group on days 1 and 7 (both P<0.01, Fig. 6a). The relative level of caspase-3 was also higher on day 7 than on day 1 after ischaemia in all groups except the sham group (P<0.01).

Discussion

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

PKC is 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 signalling pathway regulated Nrf2.13 Quesada and colleagues14 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. In an isolated rabbit heart model of ischaemic preconditioning, Zhang and colleagues reported that PKC participates in the activation of Nrf2 signalling. Nrf2 is a master redox regulator of antioxidant defence genes, including NAD(P)H:quinone oxidoreductase-1 (NQO1), glutathione S-transferase, and HO-1. 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. Plant extracts, including luteolin and ursolic acid, showed neuroprotective effects by up-regulating Nrf2 after cerebral ischaemia in a focal ischaemic animal model. Ginkgo biloba, another plant extract, also provided neuroprotection via the Nrf2/HO-1 pathway in a transient global cerebral ischaemia model. In addition, ischaemic preconditioning has been shown to have neuroprotective effects against cerebral I/R injury, also by activating Nrf2. In the present study,

![Graph and Image](image)
Sevoflurane post-conditioning provided neuroprotection by increasing Nrf2 and HO-1 expression. Similar to our result, a recent study showed that increased HO-1 expression via a PI3 K/Akt pathway was involved in sevoflurane post-conditioning-induced neuroprotection against focal cerebral I/R injury. Such findings suggest that Nrf2-mediated antioxidant enzyme production is a major neuroprotective mechanism after cerebral I/R injury.

In this study, the sevoflurane post-conditioning 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

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**Fig 3** (a) Representative photomicrographs from a single animal of TUNEL staining showing apoptotic cells in the hippocampal CA1 region on days 1 and 7 after transient global cerebral ischaemia. (b) Percentages of apoptotic cells in the hippocampal CA1 region days 1 and 7 after ischaemia (TUNEL staining). Results are given as median, inter-quartile, 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 post-conditioning group. ##P < 0.01 vs day 1. TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-uracil triphosphate biotin in situ nick end-labelling; CA1, Cornu Ammonis area 1; Sevo, sevoflurane; postC, post-conditioning; Che, chelerythrine.
A literature review indicated that direct activation of mitochondrial KATP channels, p38 mitogen-activated protein kinase activation, up-regulation of hypoxia-inducible factor-1α and HO-1 through a PI3 K/Akt pathway, and inhibition of proinflammatory cytokine production have been suggested to be involved in sevoflurane post-conditioning-induced neuroprotection after cerebral I/R injury. Sevoflurane-induced anti-excitotoxic properties also have neuroprotective effects by reducing extracellular glutamate levels and enhancing the activity of glutamate transporters. Recently, activation of the two-pore domain K+ channel TREK1, inhibition of mitochondrial permeability transition pore opening, and up-regulation of insulin-like growth factor-1 via Akt/c-Jun N-terminal kinase signalling pathways 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 signalling 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. Additionally, dose of

Fig 4 (A) Representative western blot analysis of nuclear Nrf2 from a single animal on days 1 and 7 after transient global cerebral ischaemia in rats. (B) Densitometric evaluation of nuclear Nrf2. Results are given as median, inter-quartile, 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 post-conditioning group. ##P < 0.01 vs day 1. Nrf2, nuclear factor erythroid 2-related factor 2; Sevo, sevoflurane; postC, post-conditioning; Che, chelerythrine.
Chelerythrine may incompletely block PKC, although this is unlikely since the dose of chelerythrine used in this study was relatively high the same as used in previous studies. The inhibition of apoptosis is a major mechanism in sevoflurane post-conditioning-induced neuroprotection. Anti-apoptotic effects of sevoflurane may be explained by the up-regulation 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, and also decreased caspase-3 activity. A previous study in a neurodegenerative disease model showed that attenuation of apoptotic death occurred with Nrf2 pathway modulation. Also, in a traumatic brain injury model, the apoptotic index was increased in mice when Nrf2 was deleted. 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 post-conditioning 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 post-conditioning in a rat model of transient global cerebral I/R injury. A PKC inhibitor, chelerythrine,
reduced the anti-apoptotic effect of sevoflurane post-conditioning in our study. Also consistent with our findings, a previous study demonstrated that electroacupuncture pre-treatment reduced apoptosis in rats after transient focal cerebral I/R injury, while the neuroprotective effect was abolished by a specific epsilon PKC inhibitor. Other studies have shown that the anti-apoptotic effect of ischaemic preconditioning on myocardium was lost with PKC inhibition.

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 ischaemia showed that sulforaphane, an Nrf2 inducer, increased the expression of Nrf2 at 24 h after reperfusion and decreased it at 72 h after reperfusion. Similar to their study, the expression of Nrf2 and its target antioxidant enzyme, HO-1, increased 24 h after ischaemia and declined 7 days after ischaemia in this study. The levels of Nrf2 and HO-1 on day 7 post-ischaemia were not significantly different between the control and sevoflurane post-conditioning groups in our study. Shokeir and colleagues reported that an apoptotic marker, caspase-3, continued to increase up to 48 h post-ischaemia and the level of caspase-3 was higher after 7 days, than at 1 day after ischaemia. Taken together, an antioxidant pathway may be involved in the neuroprotection of sevoflurane post-conditioning in the early phase after cerebral I/R.

Fig 6 (a) Representative western blot analysis of caspase 3 from a single animal on days 1 and 7 after transient global cerebral ischaemia. (b) Densitometric evaluation of caspase-3 protein expression. Results are given as median, inter-quartile, 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 post-conditioning group. #P < 0.05 vs the control group. ##P < 0.01 vs day 1. Sevo, sevoflurane; postC, post-conditioning; Che, chelerythrine.
injury, whereas an anti-apoptotic process may be involved in the delayed phase after ischaemia.

Chelerythrine is a potent but non-specific inhibitor of PKC. In this study, chelerythrine was i.v. administered to inhibit PKC activity before cerebral ischaemia. Cerebral I/R injury itself can activate certain types of PKC isoforms. Indeed, previous studies demonstrated that PKC isoforms, especially PKCδ and PKCγ, were activated by cerebral I/R injury. A recent study in a focal cerebral ischaemia model showed that mitochondrial KATP channels and PKCε were involved in sevoflurane preconditioning-induced neuroprotection. Another report showed that inhibition of PKCδ was involved in limb remote post-conditioning-induced neuroprotection. 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 non-specific PKC inhibitors are used, experimental results should be interpreted with caution, as they conceal the role of individual PKC isoforms, affecting inconsistency in reports on PKC function.

Our 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 in the anti-oxidative and neuroprotective effects of sevoflurane post-conditioning was not fully elucidated in this study, although other studies showed that the PKC signalling pathway was engaged in sevoflurane preconditioning-induced neuroprotection. Secondly, the extent of Nrf2 translocation from the cytosol to nucleus was not completely investigated in this study because cytoplasmic Nrf2 expression was not measured. However, previous experimental studies showed that activated nuclear Nrf2, not inactivated cytoplasmic Nrf2, played a pivotal role in inducing HO-1 activation. A recent study revealed that sevoflurane post-conditioning 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. Thirdly, we measured Nrf2, HO-1, and caspase-3 levels 1 and 7 days after ischaemia. 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 ischaemia and that apoptosis increased over time. 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 post-conditioning not only reduced apoptosis but also increased Nrf2 and HO-1 expression, which was in part blocked by chelerythrin, in the early phase after transient global cerebral ischaemia in a rat. Such results suggest that the antioxidant effect of sevoflurane post-conditioning may be responsible for neuroprotection in the early phase after cerebral I/R injury.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

Authors’ contributions
H.L. was involved in design and conduct of the study, data collection and analysis, and writing the paper. Y.H.P., E.K., and S.Y.P. participated in study design and conduct, and data collection. Y.T.J., J.W.H., and Y.J.L. participated in the study design and the data analysis, and manuscript preparation. H.P.P. helped with study design and data analysis, and completion of the manuscript. All authors approved the final manuscript.

Declaration of interest
None declared.

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References
1 Huang HC, Nguyen T, Pickett CB. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. Proc Natl Acad Sci USA 2000; 97: 12475–80
5 Kessler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007; 47: 89–116
9 Ruiz S, Pergola PE, Zager RA, Vaziri ND. Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. Kidney Int 2013; 83: 1029–41
11 Li L, Zhang X, Cui L, et al. Ursolic acid promotes the neuroprotection by activating Nrf2 pathway after cerebral ischemia in mice. Brain Res 2013; 1497: 32–9

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12 Niture SK, Jain AK, Jaiswal AK. Antioxidant-induced modification of Nrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. J Cell Sci 2009; 122: 4452–64


18 Espada S, Rojo AI, Salinas M, Cuadrado A. The muscarinic M1 receptor activation of Nrf2 through a signaling cascade that involves protein kinase C and inhibition of GSK-3beta: connecting neurotransmission with neuroprotection. J Neurochem 2009; 110: 1107–19


25 Seo JH, Park HP, Jeon YT, Lim YJ, Nam K, Hwang JW. Combined treatment with celecoxib and sevoflurane after global cerebral ischemia has no additive neuroprotective effects in rats. Br J Anaesth 2013; 110: 988–95


33 Rado P, Rojo AL, Chowdhry S, McMahan M, Hoyes JD, Cuadrado A. SCF/(beta)-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. Mol Cell Biol 2011; 31: 1121–33


35 Ma Q, He X. Molecular basis of electrophilic and oxidative defense: promises and perils of Nrf2. Pharmacol Rev 2012; 64: 1055–81


40 Tusi SK, Ansari N, Amini M, Amirabad AD, Shafiee A, Khodagholi F. Attenuation of NF-kappaB and activation of Nrf2 signaling by 1,2,4-triazine derivatives, protects neuron-like PC12 cells against apoptosis. Apoptosis 2010; 15: 738–51


46 Shokeir AA, Hussein AM, Barakat N, Abdelaziz A, Elgarba M, Awadalla A. Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf2-dependent genes by ischemic pre-conditioning and post-conditioning: new adaptive endogenous protective


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