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ORIGINAL PAPER

# Sevoflurane Post-conditioning Protects Primary Rat Cortical Neurons Against Oxygen–Glucose Deprivation/Resuscitation: Roles of Extracellular Signal-Regulated Kinase 1/2 and Bid, Bim, Puma

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**Abstract** Temporal post-conditioning to induce neuroprotection against brain ischemia–reperfusion injury insult is considered to be an effective intervention, but the exact mechanisms of sevoflurane post-conditioning are poorly understood. Extracellular signal-related kinases 1/2 (Erk1/2) play a pivotal role in the cell growth and proliferation. The essential axis of activator Bid, Bim, Puma (BH3s) and BAX, BAK in activating the mitochondrial death program might offer common ground for cell death signal. We hypothesized that, sevoflurane post-conditioning might inhibit the expression of Bid, Bim and Puma and is activated by phosphor-Erk1/2 to reduce neuronal death. To test this hypothesis, we exposed primary cultured cortical neurons to oxygen–glucose deprivation for 1 h and resuscitation for 24 h (OGD/R). The assays of MTT, propidium iodide uptake, JC-1 fluorescence and western blot demonstrated that OGD/R exposure reduced cell viability, increased cell death, decreased mitochondrial membrane potential and the expressions of Bid, Bim, and Puma. Inhibition of Erk1/2 phosphorylation could partially attenuate 2 % of sevoflurane post-conditioning mediated increase in neuronal viability and mitochondrial membrane potential, and also a decrease in cell death and expression of Bid, Bim and Puma after OGD/R treatment. The results demonstrated that, the protection of sevoflurane post-conditioning markedly reducing death of cortical neurons exposed to OGD/R could be correlated with down-regulation of Bid, Bim and Puma expression mediated by phosphorylation/activation of Erk1/2.

**Keywords** Sevoflurane · Neuroprotection · Extracellular signal-related kinases 1/2 · Bid · Bim · Puma

## Introduction

It is difficult to predict when brain ischemia–reperfusion injury (IRI) happens, so post-conditioning in time to induce neuroprotection against IRI insult is considered to be an effective intervention [1]. Sevoflurane, as an inhalational anesthetic agent is frequently administered for general anesthesia, and the neuroprotective effects of sevoflurane post-conditioning in vivo and in vitro have been demonstrated in some studies [1, 2]. Although there is much evidence that sevoflurane post-conditioning has potential neuroprotective ability, the mechanisms are poorly understood [3, 4].

Mammalian cell death due to necrosis and apoptosis induced by ischemia–reperfusion is strongly dependent on mitochondria [5, 6]. The Bcl-2 family protein is a crucial checkpoint that controls the process of apoptosis in mitochondria [7]. Bid, Bim and Puma are regarded as BH3-only protein (BH3s) in Bcl-2 family, which could activate Bax and Bak to release cytochrome c-mediated activation of caspase in response to diverse death signal [8]. In both in vivo and in vitro studies, extracellular signal-related kinases 1 and 2 (Erk1/2) have been demonstrated to be crucially involved in modulating brain cell death and survival after ischemia [9, 10]. Interestingly, sevoflurane post-conditioning protects isolated rat hearts against myocardial

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I/R injury and inhibits cell death via activation of Erk1/2 pathway [11]. Additionally, it is reported that inhibition of Erk1/2 signaling induced up-regulation of Bid, Bim and Puma is correlated with cell death induction in different cancer cell lines [12–16].

So far, little attention has been paid to the effects of sevoflurane post-conditioning on the changes of Erk1/2 and of Bid, Bim, Puma expressions. We questioned whether sevoflurane post-conditioning might down-regulate Bid, Bim and Puma expressions mediated by phosphorylation/activation of Erk1/2 to attenuate neuronal death. In this study, we therefore exposed primary rat cortical neurons to oxygen–glucose deprivation and resuscitation (OGD/R) stimulating ischemia and reperfusion in vitro, and then investigated the effects of OGD/R and sevoflurane post-conditioning. We demonstrated that the neuroprotection of sevoflurane post-conditioning could improve mitochondrial dysfunction via down-regulation of Bid, Bim and Puma expressions mediated by phosphorylation/activation of Erk1/2.

## Methods

All protocols we followed in our study was approved by the animal research committee of The First Affiliated Hospital of China Medical University.

### Cell Culture

Primary cultures of cortical neurons from newborn Sprague-Dawley rats were used as previously described [17]. Under sterile conditions, cortical tissue from neonates was dissociated in 5 mM-cysteine (Sigma, St. Louis, MO, USA), 10 units/ml papain (ICN, Irvine, CA, USA) and 0.01 % DNase I (Sigma) by trituration at 37 °C. Dissociated neurons were washed in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, GrandIsland, NY, USA), centrifuged and gently resuspended in DMEM medium with serum. After 24 h, the neurons were washed with phosphate-buffered saline (PBS) and gently resuspended in neuron-defined serum-free Neuorbasal medium (Gibco-BRL) supplemented with B27 (Gibco-BRL), 0.5 mM L-glutamine (Gibco-BRL), and 2 μg/mL gentamycin. The cells were plated at $8.5 \times 10^5$ cells/mL onto 100 mm culture dishes coated with 0.1 mg/mL poly-L-lysine (Sigma). Neuronal cultures were maintained in a humidified atmosphere of 95 % air–5 % $CO_2$ at 37 °C. On day in vitro (DIV) 4, one third of the culture medium was removed and replaced with fresh Neuorbasal medium/B27/glutamine/gentamycin containing cytosine arabinoside (10 mM, Sigma), and on DIV 8 one half of the culture medium was replaced with Neuorbasal medium/B27. Cortical tissue from neonatal rat harvested on DIV 8 was subjected to staining with NeuN to access the percentage of neurons in the primary cultures. For quantitative measurement of the percentage of neuronal and astrocytic cells, we performed the NeuN/Glial fibrillary acidic protein (GFAP) fluorescence assay similar to our previous studies. Briefly, after fixing with 4 % paraformaldehyde, 4 % sucrose, and 0.1 M PBS for 30 min at 4 °C, neuronal cultures were washed, permeabilized with 0.3 % Triton X-100, and blocked with 10 % serum. Cells were cultured in anti-NeuN monoclonal antibody (1:1000; Chemicon, Temecula, CA, USA) and anti-GFAP polyclonal antibody (1:4000; Dako, Carpinteria, CA, USA) with 3 % serum at 4 °C overnight; then with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 546 secondary fluorescent antibodies (Molecular Probes, Eugene, OR, USA). The percentage of NeuN-positive neurons and GFAP-positive astrocytes was determined by counting the number of NeuN- and GFAP-positive cells in eight separate fields over two wells. Purity of neuronal cultures was 86 % neurons and 11 % astrocytes [18, 19].

### OGD/R and Sevoflurane Post-conditioning

On DIV 8, cultured cortical neurons were exposed with OGD/R experiments as described previously [20]. Neuronal cultures were washed with PBS and placed in glucose-free balanced salt solution (BSS; 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM $NaH_2PO_4$, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.02 mM glycine, and 2 mg/L phenol red) that was flushed with 100 % $N_2$ for 30 min to remove oxygen in the solution. The neuronal cultures were then directly placed in an anaerobic incubator (Thermo, Waltham, MA, USA) containing 100 % $N_2$ at 37 °C. After 90 min in OGD, the cultures were removed from the anaerobic incubator, immediately washed three times with BSS before adding an equal volume of Neuorbasal medium/B27/glutamine/gentamycin and incubated in a standard cell incubator containing 5 % $CO_2$, 95 % air balance, 98 % humidity at 37 °C for 24 h. In the sevoflurane post-conditioning protocols, neuronal cultures were exposed immediately in a Billups-Rothenburg chamber that was bubbled with different concentrations of sevoflurane in 95 % air–5 % $CO_2$ at 6 L/min for 7–8 min after OGD [21]. Then the chamber was kept tightly sealed for 1 h at 37 °C and the concentrations of sevoflurane in exhaust gas from the chamber was confirmed by the Datex™ infrared analyzer (Capnomac, Helsinki, Finland). After 1 h of sevoflurane post-conditioning treatment, neurons were exposed in a standard cell incubator containing 5 % $CO_2$, 95 % air balance, 98 % humidity at 37 °C for 23 h. Neuronal cultures were added with PD98059 (a mitogen-activated protein kinase (MAPK) kinase (MEK)

inhibitor) (Selleck Chemicals, Houston, TX, USA) at a final concentration of 30 μM for 1 h prior to treatment with OGD/R according to a previously described protocol [22]. Control cell cultures not applied for OGD were exposed to BSS containing 6 mM glucose, were not bubbled with $N_2$, and incubated in the standard environment.

### Assessment of Neuronal Viability

For quantitative measurement of neuronal viability, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After the cells were incubated with 150 μl MTT (0.5 mg/mL), the formazan accumulated in living cells was dissolved in 100 μl DMSO. Optical density (OD) values were measured at 570 nm with an automatic microplate reader (Bio-Tek, Winooski, VA, USA). Survival of control was defined as 100 % [23].

### Assessment of Neuronal Injury

For quantitative measurement of neuronal injury, we used the lactate dehydrogenase (LDH) release assay. LDH absorbance (492 nm) values from control were subtracted from sevoflurane post-conditioning of different concentrations and cell injury values were expressed as a percentage of total LDH (determined for each experiment by assaying the supernatant of duplicated cultures after 30 min of exposure to 1 % Triton X-100) caused by different concentrations of sevoflurane [17, 18].

### Assessment of Neuronal Death

For quantitative measurement of neuronal death, we used the propidium iodide uptake assay. The neurons were cultured with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (5 g/mL) for 5 min, and fixed in 4 % paraformaldehyde (PFA). After washing with PBS, nuclei were counterstained with Hoechst33342 (10 μg/mL) (Invitrogen, Grand Island, NY, USA). The number of PI-positive cells was counted in at least three separate experiments per treatment condition without the knowledge of treatment history [18].

### Assessment of Mitochondrial Membrane Potential

For quantitative measurement of mitochondrial membrane potential (MMP), we used JC-1 fluorescence assay. Briefly, primary cortical neurons were incubated with 5 μM JC-1 at 37 °C for 30 min in the dark. After washing with PBS twice, images were captured under microscope (Leica, Germany), and then analyzed by image analysis software (Bio-Rad, Hercules, CA, USA). Mitochondrial depolarization of $\Delta\psi m$ was expressed by the increase of red/green fluorescence intensity ratio [24].

### Real-Time PCR Assessment of Bid, Bim and Puma mRNA Expression

Real-time PCR was performed as described in our previous study [18]. Briefly, total RNA was extracted from neuronal cultures and 500 ng of RNA in each group was reversely transcribed into cDNA using oligo dT and superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Real-time PCR was tested on a 7500 Fast Realtime PCR System (Applied Biosystems, Irvine, CA, USA) with Power SYBR Green (Applied Biosystem). The expression of Bid, Bim and Puma was normalized by a reference gene-Glyceraldehyde 3-phosphate dehydrogenase. The PCR mixtures were pre-heated at 50 °C for 2 min and then at 95 °C for 10 min to activate the Ampli Taq Gold DNA polymerse; then all reactions were subjected to 40 cycles of amplification (denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s). Finally, the PCR reactions were extended for 10 min at 60 °C. Reactions were performed in triplicate and the results were plotted, and the $\Delta\Delta C_t$ method was used to calculate relative expressions of Bid, Bim and Puma mRNA. The Primer Sequences for Bid, Bim, and Puma were

Bid: 5′-ATGGGACAGTGGTGCAGTTTT-3′ (F)
5′-AATCTTTCACTTCTCTAACTGCTCAACA-3′ (R)
Bim: 5′-ATCTCAGAGCAATGGCTTCC-3′ (F)
5′-ATTCGTGGGTGGTCTTCG-3′ (R)
Puma: 5′-TCAACGCACAGTACGAGCG-3′ (F)
5′-CATGATGAGATTGTACAGGACCC-3′ (R)

### Western Blot

Western blot analysis was performed as we described previously [18, 25]. Rabbit anti-rat polyclonal anti-Bid, anti-Bim, anti-Puma (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p44/42 MAP kinase (Thr202/Tyr204) antibody, phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies. The blots were incubated at 37 °C for 1 h. Then anti-rabbit antibody (1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a secondary antibody and the blots were incubated at 25 °C for 1 h. Protein bands were examined by western blot detection system (Amersham Biosciences, Piscataway, NJ, USA) with ECL. Blots were quantified using Quantify One Analysis Software (Bio-Rad Laboratories, USA). The relative expression level of proteins was normalized to β-actin.

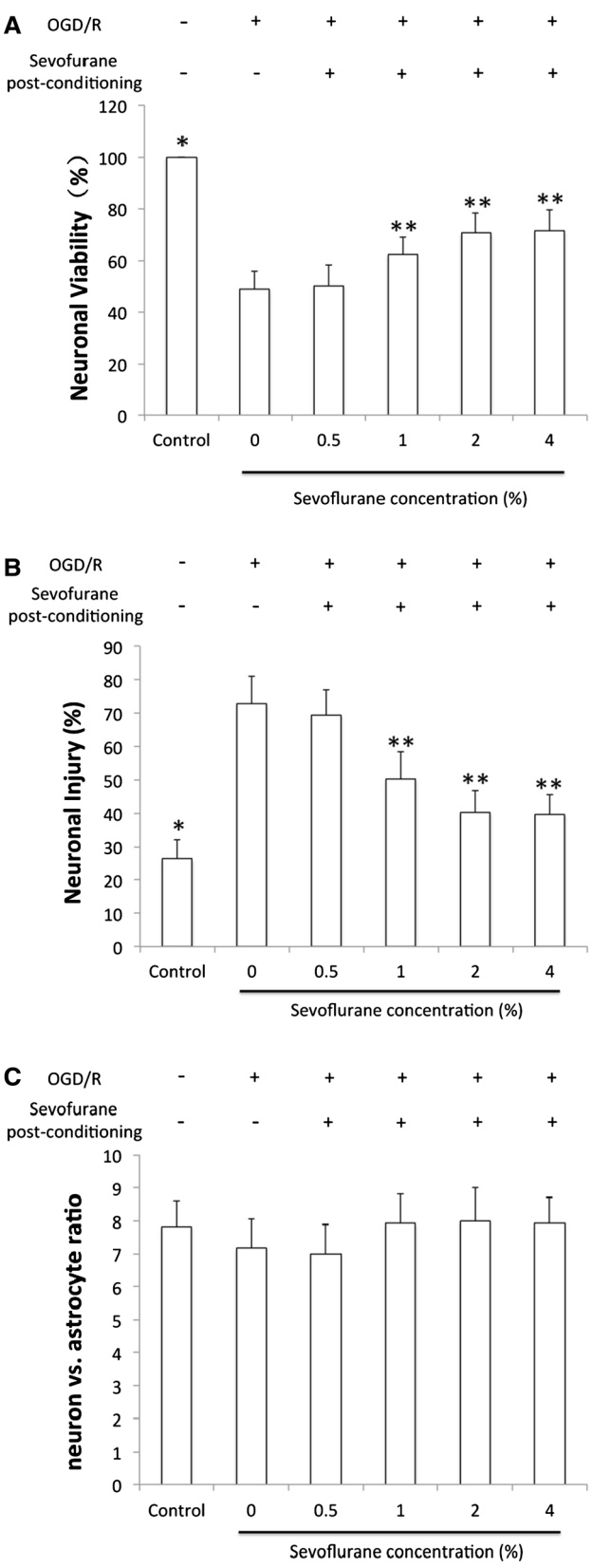


**Fig. 1** Effects of various concentrations of sevoflurane post-conditioning on the viabilities (**a**), injury (**b**), neuron versus astrocyte ratio (**c**) of oxygen–glucose deprivation and resuscitation (OGD/R) neurons. The rat cortical neurons were exposed to OGD for 90 min, incubated with different concentrations of sevoflurane for 1 h, and returned to control condition for 23 h. Results are presented as the percentage of control neurons. Control = neurons without OGD/R or sevoflurane treatment. The data were expressed as mean ± SD (n = 6/group), $*P < 0.05$ versus 0 % sevoflurane post-conditioning, $**P < 0.05$ versus control

## Statistics

All experiments were performed for at least three independent assays in different neuronal preparations to get comparable results for statistical analysis and the data are expressed as mean ± SD. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni–Dunn tests. $P < 0.05$ was considered statistically significant.

## Results

### 2 % Sevoflurane Post-conditioning Induced Highest Increase in Viability of OGD/R Neurons

The effects of different concentrations of sevoflurane post-conditioning in viability of OGD/R neurons were investigated by MTT and LDH assay. The rat cortical neuronal cultures were exposed to OGD for 90 min, then treated with 0, 0.5, 1, 2, 4 % sevoflurane for 1 h, and returned to control conditions for 23 h. The results of MTT assay showed that neuronal viability increased with 1, 2 and 4 % sevoflurane post-conditioning (Fig. 1a). It was also found that, post-treatment with 2 % sevoflurane induced a significant decrease in LDH release in the OGD neurons by LDH assay (Fig. 1b). Therefore, 2 % sevoflurane post-conditioning was used in the following experiments because of the highest value of neuronal viability. Additionally, according to the result of neuron/astrocytes ratio, it was found that there was no significant difference between the groups as mentioned above (Fig. 1c).

### The Neuroprotection of Sevoflurane Post-conditioning Could be Partially Reduced by Inhibition of Erk1/2 Phosphorylation

As shown in Fig. 2, western blot analysis indicated that the integrated density value ratio of p-ErK (phosphorylated Erk, active Erk)/t-Erk (total Erk, including phosphorylated

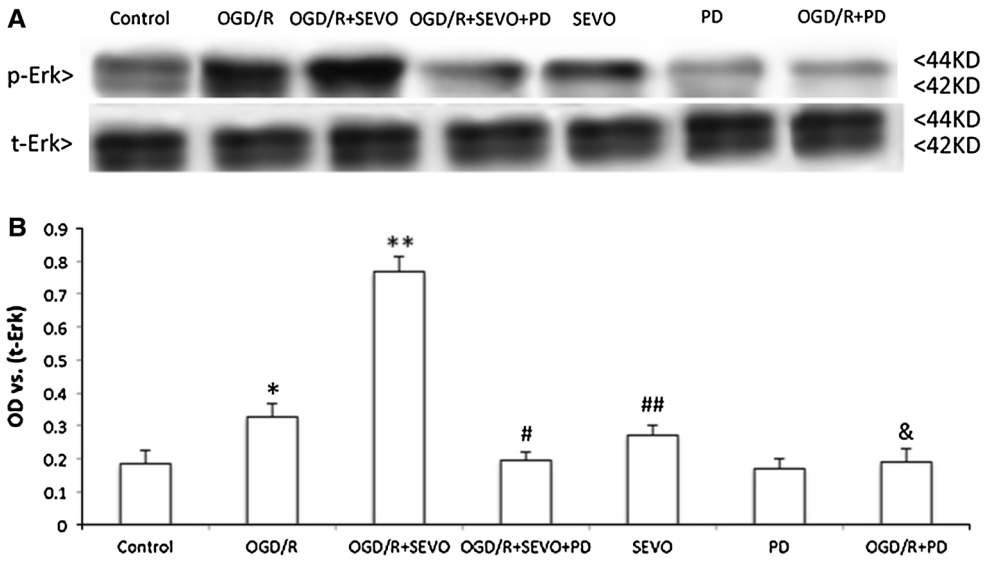


**Fig. 2** Changes of phosphorylated Erk1/2 in the primary rat cortical neuron cultures caused by indicated stimuli. **a** Representative western blot of phosphorylated Erk1/2 (p-Erk) and total Erk1/2 (t-Erk). **b** Optical density (OD) value of phosphorylated Erk1/2 evaluated by western blot analysis. Data are presented as mean ± SD (n = 5/group). Control: neurons without OGD/R or sevoflurane treatment, OGD/R: neurons incubated with OGD for 90 min, and returned to normal condition for 24 h, OGD/R + SEVO: neurons incubated with OGD for 90 min, exposed to 2 % sevoflurane for 1 h, and returned to normal condition for 23 h, OGD/R + SEVO + PD: neuronal cultures were added into PD98059 for 1 h prior to treatment with OGD, incubated with OGD for 90 min, exposed to 2 % sevoflurane for 1 h, and returned to normal condition for 23 h, SEVO: neurons incubated with 2 % sevoflurane for 1 h, PD: neuronal cultures were added into PD98059 for 25 h, OGD/R + PD = neuronal cultures were added into PD98059 for 1 h prior to treatment with OGD, incubated with OGD for 90 min, and returned to normal condition for 24 h. $^{*}P < 0.05$ versus control. $^{**}P < 0.05$ versus OGD/R. $^{\#}P < 0.05$ versus OGD/R + SEVO. $^{\#\#}P < 0.05$ versus control. $^{\&}P < 0.05$ versus OGD/R

and dephosphorylated Erk) was significantly increased with OGD/R or OGD/R plus sevoflurane post-conditioning treatment compared with control ($P < 0.05$ vs. control; Fig. 2). However, further analysis of the results showed that, p-ErK/t-ErK was significantly higher after sevoflurane post-conditioning treatment than OGD/R alone ($P < 0.05$ vs. OGD/R; Fig. 2), while PD98059 largely reversed it ($P < 0.05$ vs. OGD/R + SEVO; Fig. 2). Additionally, compared with control, the values of p-ErK/t-ErK in the neuronal cultures exposed to sevoflurane alone were higher ($P < 0.05$ vs. Control; Fig. 2). It was also indicated that, p-ErK/t-ErK in the neuronal cultures with PD98059 treatment at the onset of OGD/R, significantly decreased compared with OGD/R exposure alone ($P < 0.05$ vs. OGD/R; Fig. 2).

The percentage of cell viability was reduced to 48.7 ± 7.0 % in cultures exposed to OGD/R compared with control cultures ($P < 0.05$ vs. control, Fig. 3) by MTT assay. The result of propidium iodide (PI) uptake assay showed that, neuronal cell death was increased by PI-positive cells from 7.3 ± 1.5 % in control to 35.7 ± 6.0 % after OGD/R exposure ($P < 0.05$ vs. control, Fig. 4). But the living cells were increased significantly to 70.3 ± 7.8 % and PI-positive cells were attenuated (15.7 ± 2.5 %) after sevoflurane post-conditioning treatment ($P < 0.05$ vs. OGD/R, Figs. 3, 4). Treatment with PD98059 decreased the neuronal cell viability (57.3 ± 5.7 %) and increased the neuronal cell death (24.0 ± 2.6 %) in cultures exposed to OGD/R plus sevoflurane post-conditioning treatment ($P < 0.05$ vs. OGD/R + SEVO, Figs. 3, 4). In addition, it was indicated that there were no significant difference in neuronal cell viability and cell death between control and sevoflurane or PD98059 treatment alone. However, compared with OGD/R exposure alone, neuronal cultures with OGD/R plus PD98059 treatment revealed a decrease in cell viability and an increase in cell death ($P < 0.05$ vs. OGD/R, Figs. 3, 4).

## The Neuroprotection of Sevoflurane Post-conditioning Could be Related to Decrease in Bid, Bim, Puma mRNA and Protein

The effects of 2 % sevoflurane post-conditioning on the mitochondrial membrane potential (MMP) of OGD/R neurons were investigated by JC-1 fluorescence assay. Typical distributions of red and green fluorescent signals were observed in primary cortical neuron cultures. Our result showed that, the MMP level was significantly decreased after OGD/R treatment compared with control, as indicated by an increase in green fluorescence and a decrease in red fluorescence ($P < 0.05$ vs. control, Fig. 5). Moreover, with sevoflurane post-conditioning treatment,

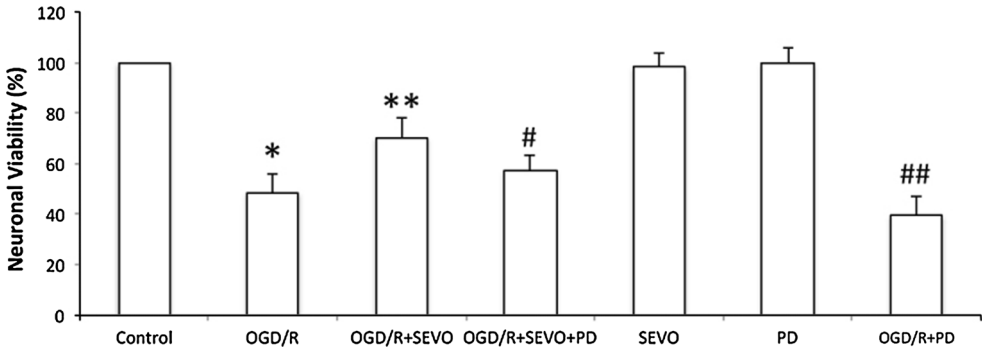


**Fig. 3** Inhibition of Erk1/2 phosphorylation reduced sevoflurane post-conditioning induced increases of primary rat cortical neuronal viability after oxygen–glucose deprivation and resuscitation (OGD/R) treatment. MTT revealed that the viability of primary cultured cortical neurons exposed to OGD/R was decreased. Sevoflurane post-conditioning partially restored viability, while PD98059 reversed it. Data are presented as mean ± SD (n = 12/group). Control, OGD/R, OGD/R + SEVO, OGD/R + SEVO + PD, SEVO, PD, and OGD/R + PD denotation were described previously. $^{*}P < 0.05$ versus Control. $^{**}P < 0.05$ versus OGD/R. $^{\#}P < 0.05$ versus OGD/R + SEVO. $^{\#\#}P < 0.05$ versus OGD/R

the MMP level increased after OGD/R exposure ($P < 0.05$ vs. OGD/R, Fig. 5). Our results also showed that, there was no significant difference of MMP between control and 2 % sevoflurane or PD98059 treatment alone. In addition, it was showed that, the increase in MMP level induced by sevoflurane post-conditioning was partially eliminated by PD98059 ($P < 0.05$ vs. SEVO, Fig. 5). Moreover, it was also showed that OGD/R plus PD98059 treatment decreased MMP level compared with OGD/R exposure alone ($P < 0.05$ vs. OGD/R, Fig. 5).

Real-time PCR and Western blot analysis indicated that, compared with baseline in control, the mRNA and protein expressions of Bid, Bim and Puma were elevated after OGD/R or OGD/R plus sevoflurane post-conditioning treatment ($P < 0.05$ vs. control; Fig. 6). With further analysis, sevoflurane post-conditioning for 1 h after OGD partially restored the increase ($P < 0.05$ vs. OGD/R; Fig. 6). Additionally, the mRNA and protein expressions of Bid, Bim and Puma in control cultures were not different with 2 % sevoflurane or PD98059 treatment alone. Moreover, treatment with PD98059 of the OGD/R + SEVO group increased the mRNA and protein expressions of Bid, Bim and Puma ($P < 0.05$ vs. OGD/R + SEVO, Fig. 6). But the cultures exposed to OGD/R plus PD98059 revealed an increase in Bid, Bim and Puma expression compared with OGD/R treatment alone ($P < 0.05$ vs. OGD/R, Fig. 6).

## Discussion

We showed that OGD/R exposure to primary cultured cortical neurons resulted in a significant decrease in neuronal cell viability (as assessed by MTT assay), an increase in neuronal cell death (as assessed by PI assay), and a decrease in mitochondrial membrane potential (assessed by JC-1 fluorescence assay). However, consistent with previous studies, neuronal cell viability was increased, and neuronal cell death and mitochondrial dysfunction were significantly attenuated by sevoflurane post-conditioning [1]. PD98059, which inhibits phosphorylation/activation of Erk1/2 in neurons, could partially eliminate neuroprotection induced by sevoflurane post-conditioning. Additionally, OGD/R induced significant increase in expressions of Bid, Bim, Puma mRNA/protein, whereas increase in Bid, Bim and Puma expressions were down regulated by sevoflurane post-conditioning, but PD98059 treatment reversed it. Thus, we demonstrated that, the neuroprotective effects of sevoflurane post-conditioning could be correlated with the decrease in Bid, Bim and Puma expression mediated by phosphorylation/activation of Erk1/2.

The concentration of sevoflurane post-conditioning set to 2 % is usually used as clinically relevant concentration, which significantly reduces the OGD and stimulates reperfusion-induced neuronal cell death. However, consistent with previous studies, exposure to 2 % sevoflurane post-conditioning could significantly increase neuronal cell viability and reduced neuronal cell death compared with 1 or 4 % [1]. In addition, with extending the post-conditioning exposure time, the toxicity of sevoflurane to neuron was gradually presented, so post-conditioning for 1 h after OGD was carried out to apply [26, 27]. Moreover, from the result of neuron/astrocyte ratio, no significant changes were found between control and different concentrations of sevoflurane. It might be indicated that sevoflurane post-conditioning could provide familiar degree of protection on neuron versus glia [19].

From the results of western blot, phosphorylation of Erk1/2 significantly increased in the primary cortical neuron cultures exposed to OGD/R or OGD/R plus sevoflurane

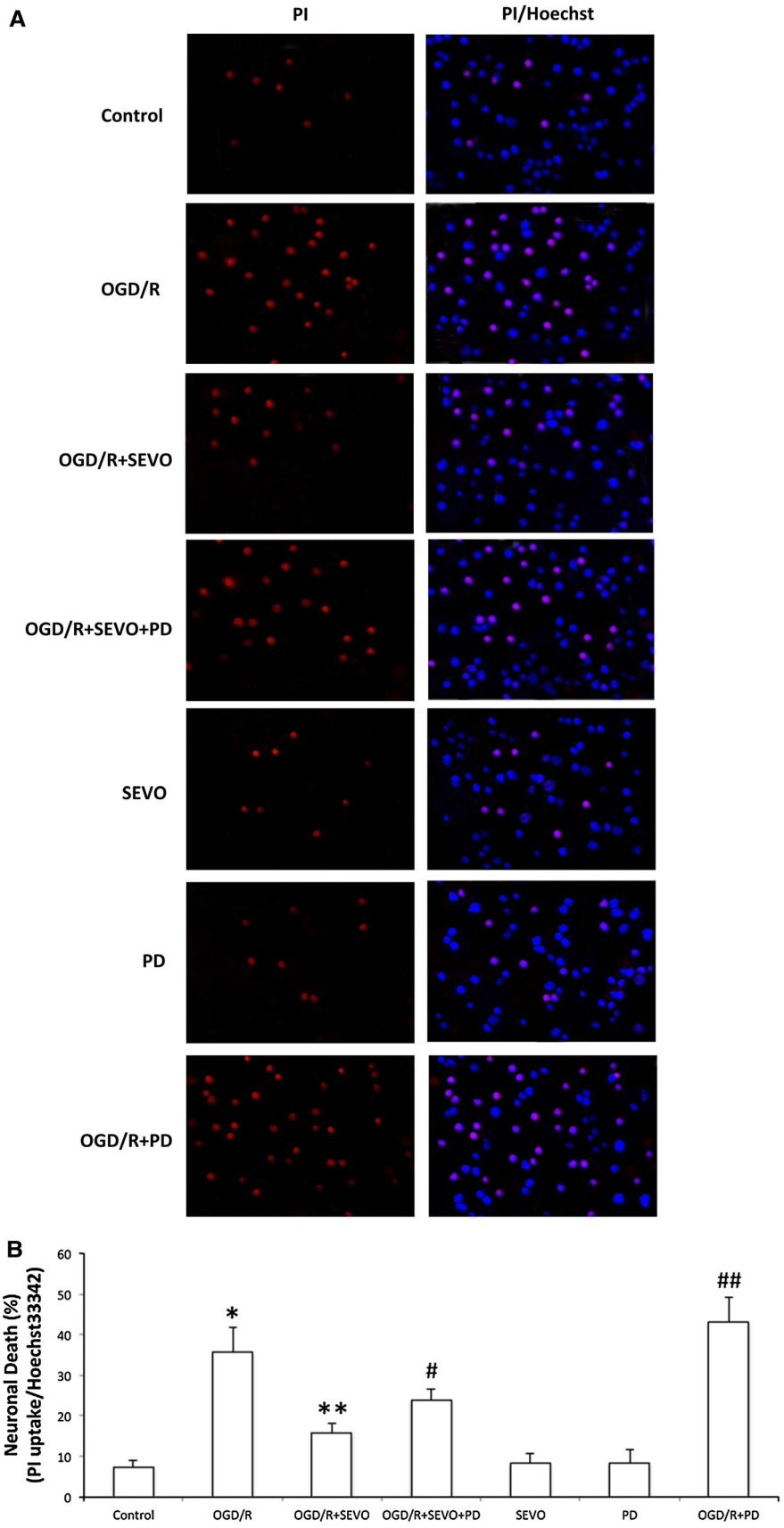


**Fig. 4** Inhibition of Erk1/2 phosphorylation reduced sevoflurane post-conditioning induced decrease in primary rat cortical neuronal death after oxygen–glucose deprivation and resuscitation (OGD/R) treatment by propidium iodide (PI) staining assay. **a** PI-positive cells (PI *red*; Hoechst 33342 *blue*) increased after OGD/R indicating increased neuronal apoptosis, but the value was decreased after sevoflurane post-conditioning treatment, while PD98059 reversed it. Data are presented as mean ± SD (n = 5/group). **b** Control, OGD/R, OGD/R + SEVO, OGD/R + SEVO + PD, SEVO, PD and OGD/R + PD denotation were described previously. $^{*}P < 0.05$ versus control. $^{**}P < 0.05$ versus OGD/R. $^{\#}P < 0.05$ versus OGD/R + SEVO. $^{\#\#}P < 0.05$ versus OGD/R (Color figure online)

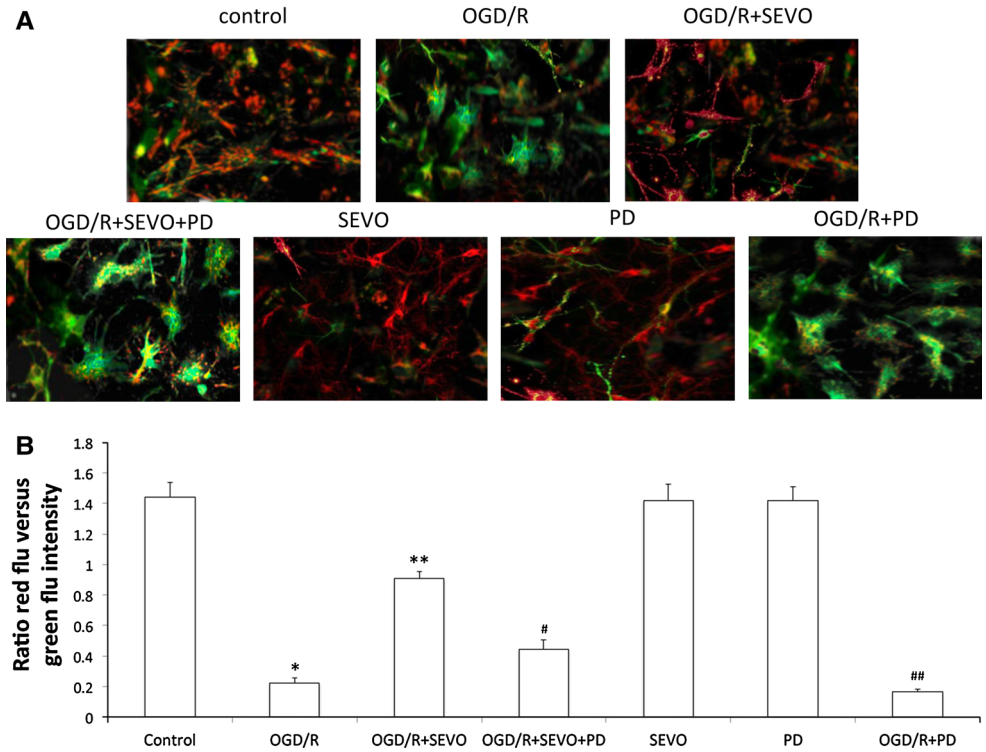


**Fig. 5** Inhibition of Erk1/2 phosphorylation reduced sevoflurane post-conditioning induced changes of primary rat cortical neuronal mitochondrial membrane potential after oxygen–glucose deprivation and resuscitation (OGD/R) treatment by JC-1 fluorescence staining assay. **a** Ratio of *red*/*green* fluorescence represented by confocal images decreased after OGD/R indicating decreased mitochondrial membrane potential, but the value was increased after sevoflurane post-conditioning treatment, while PD98059 reversed it. Control, OGD/R, OGD/R + SEVO, OGD/R + SEVO + PD, SEVO, PD and OGD/R + PD denotation were described previously. **b** Data are presented as mean ± SD (n = 5/group), $^{*}P < 0.05$ versus control. $^{**}P < 0.05$ versus OGD/R. $^{\#}P < 0.05$ versus OGD/R + SEVO. $^{\#\#}P < 0.05$ versus OGD/R (Color figure online)

post-conditioning treatment. Our further analysis indicated that, sevoflurane post-conditioning seemed to induce more phosphorylation/activation of Erk1/2. Our study also showed that, OGD/R treatment induced significant decrease in neuronal cell viability and increase in cell death, while sevoflurane post-conditioning could restore it. However, with inhibition of phosphorylation of Erk1/2 induced by PD98059, the neuroprotective effects of sevoflurane post-conditioning were largely eliminated. The special inhibition of the MAPK signal transduction pathway by means of PD98059, a MAPK kinase (MEK, upstream from Erk1/2) inhibitor, could prevent phosphorylation of Erk1/2 (at $Ser^{199}/Ser^{202}$) and shut down its activity. Phosphorylation of Erk1/2 was inhibited by 53 % in cells incubated in the presence of PD98059 at a final concentration of 20 μM [22]. In our study, PD98059 was added to the culture medium at a final concentration of 30 μM for 1 h as previously described applications in neurons [22, 28]. Interestingly, sevoflurane exposure alone increased phosphorylation of Erk1/2 slightly, but PD98059 treatment alone could significantly attenuate the increase of phosphorylation of Erk1/2 induced by OGD/R. It has been found that, sevoflurane post-conditioning inhibits myocardial cell death via activation of Erk1/2 pathway [11, 29]. Our study indicated that, sevoflurane post-conditioning could protect primary rat cortical neurons against OGD/R mediated by activation of Erk1/2.

Further, it was also found that, mitochondrial dysfunction presented by the changes in mitochondrial membrane potential was observed after OGD/R, while sevoflurane post-conditioning attenuated this effect. It was suggested that, the neuroprotection of sevoflurane post-conditioning could be correlated with mitochondrial-induced neuronal cell death. Interestingly, with sevoflurane post-conditioning treatment, the mRNA and protein expressions of Bid, Bim and Puma were down regulated after OGD/R exposure. But with inhibition of phosphorylation of Erk1/2, the decrease in Bid, Bim and Puma expression was partially eliminated. The less strong effect in increasing mRNA/protein expression of Bid, Bim and Puma induced by the MAPK pathway inhibitor PD98059 might be related with other activating/inhibiting signal pathway like hypoxia-inducible factor-1α (HIF-1α) activated by sevoflurane post-conditioning [2]. Some studies showed that HIF-1α inhibits

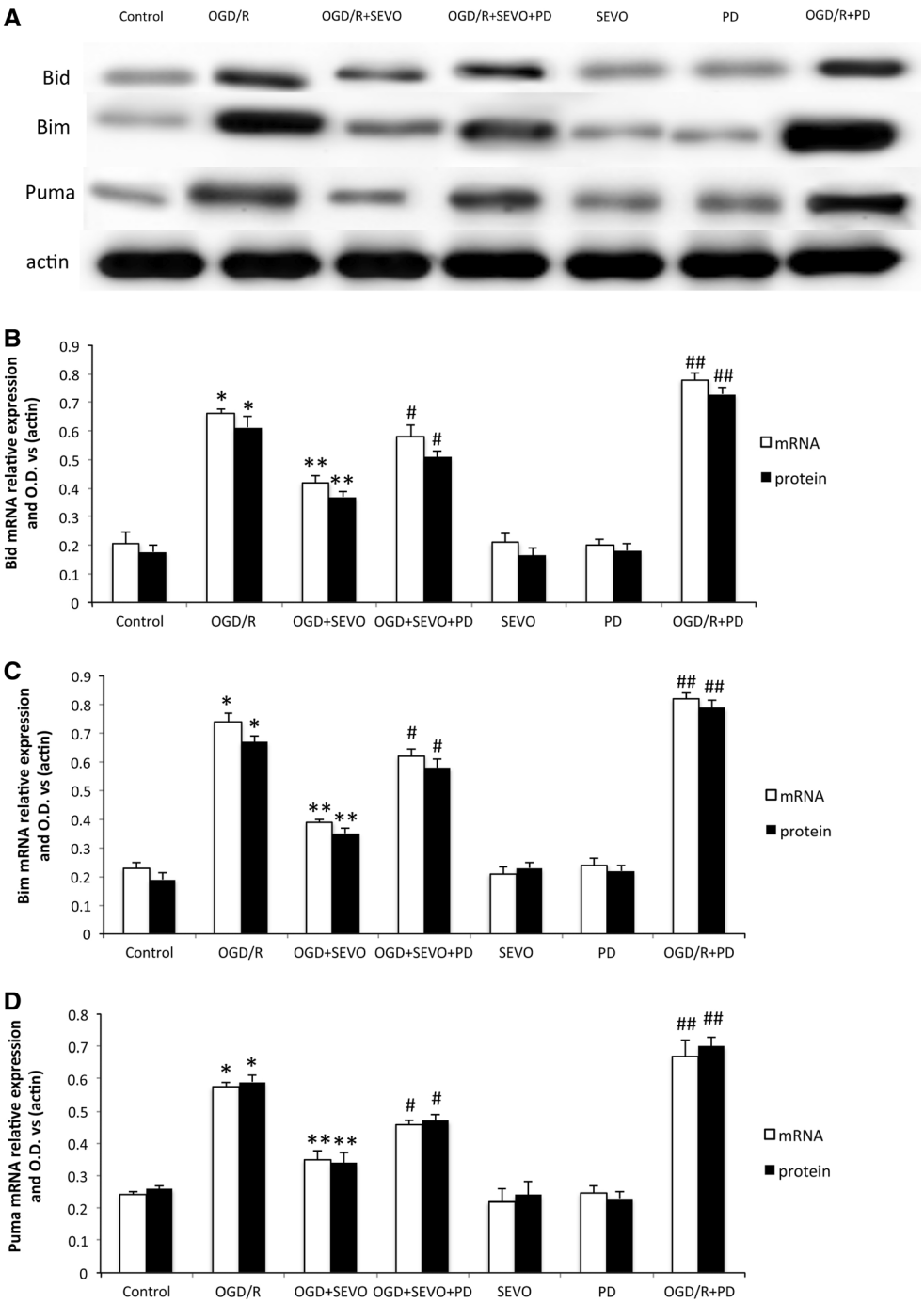


**Fig. 6** Inhibition of Erk1/2 phosphorylation reduced sevoflurane post-conditioning induced decreases of Bid, Bim and Puma expression in primary rat cortical neuron culture after oxygen–glucose deprivation and resuscitation (OGD/R) treatment. **a** Representative western blot of Bid, Bim and Puma. Data are presented as mean ± SD (n = 6/group). **b–d** mRNA relative expression and optical density (OD) value of Bid, Bim, Puma evaluated by real-time PCR and western blot analysis. Control, OGD/R, OGD/R + SEVO, OGD/R + SEVO + PD, SEVO, PD, and OGD/R + PD denotation were described previously. $^{*}P < 0.05$ versus control. $^{**}P < 0.05$ versus OGD/R. $^{\#}P < 0.05$ versus OGD/R + SEVO. $^{\#\#}P < 0.05$ versus OGD/R

transcriptions of Bid, Bim and Puma gene that may help cells to adapt to hypoxic/ischemic circumstances [30–33]. An essential axis of activation of Bid, Bim, Puma-BAX and BAK was correlated with activating the mitochondrial death program, which could offer common ground for therapeutic interventions [8]. Deficiency of Bim/Puma prevents ER-stress-induced BAX/BAK activation and death [34]. Some studies proposed that, the activation of BAX and BAK occurs by default as long as all the anti-apoptotic Bcl-2 proteins are neutralized by BH3s, based on the observation that BAX- or BAK-dependent apoptosis proceeds in the absence of Bid and Bim [35]. BAX- or BAK-dependent permeabilization of the MOM can be directly activated by translated Puma protein in vitro [34, 36, 37]. The remaining BH3s including BAD, NOXA, BMF, HRK and BIK/BLK do not activate BAK/BAX directly, but instead prevent the anti-apoptosis Bcl-2 members from sequestering the activator [8]. It is

demonstrated that, the neuroprotective effect of sevoflurane post-conditioning might be related to the decrease in Bid, Bim and Puma expressions via Erk1/2.

In summary, this study confirmed that sevoflurane post-conditioning induced a neuroprotective effect on primary cultured cortical neurons in an in vitro model of ischemia and reperfusion. This beneficial effect may be associated with the activation of Erk1/2 phosphorylation to decreases the expression levels of Bid, Bim and Puma which could trigger the death of mitochondria and promotes cell death cascade.

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