

Sevoflurane alleviates oxygen-glucose deprivation/reoxygenation-induced injury in HT22 cells through regulation of the PI3K/AKT/GSK3 β signaling pathway

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Abstract. Sevoflurane (Sev), a volatile anesthetic, has been reported to exhibit beneficial effects on different ischemia/reperfusion (I/R)-injured organs. However, the neuroprotective effect of Sev on cerebral I/R injury is poorly understood. In the present study, the effects of Sev on HT22 cells exposed to oxygen-glucose deprivation/reperfusion (OGD/R) injury are investigated. The present study demonstrated that OGD/R suppressed the cell viability and increased lactate dehydrogenase (LDH) release from the cells, and these effects were attenuated by Sev treatment. The results also demonstrated that Sev alleviated OGD/R-induced cell apoptosis via flow cytometry and caspase-3 activity determination. Biochemical analysis results revealed that Sev significantly protected against OGD/R-induced oxidative stress by reducing ROS generation and improving antioxidant defense markers. Western blot analysis demonstrated that Sev reactivated the PI3K/AKT/glycogen synthase kinase-3 β (GSK3 β) signaling pathway, which was inhibited by OGD/R. In addition, wortmannin, a selective PI3K inhibitor was used to investigate the underlying pathways. Notably, the neuroprotective effect of Sev on apoptosis and reactive oxygen species production was found to be suppressed by wortmannin. Collectively, these results demonstrated that Sev may protect neuronal cells against OGD/R-induced injury through the activation of the PI3K/AKT/GSK3 β signaling pathway. The findings from the present study provide a novel insight into understanding the neuroprotective effect of Sev on cerebral I/R injury.

Introduction

Cerebral ischemia/reperfusion (I/R) injury, one of the most common types of stroke, often causes a variety of cerebrovascular and cardiovascular diseases that lead to human disability and mortality worldwide (1,2). Nerve cell injury and apoptosis are considered the first steps in the pathogenesis of cerebral I/R injury (3). Once cerebral I/R occurs, a large number of oxygen free radicals and acute inflammatory reactions are induced, resulting in apoptosis and therefore aggravating the ischemic brain tissue injury (4). Therefore, extensive research is needed to find effective anti-apoptosis and anti-oxidative stress agents that can inhibit apoptosis and oxidative stress, ameliorating cerebral I/R injury.

Sevoflurane (Sev), a volatile anesthetic, has potential therapeutic effects on I/R-induced injury, especially towards the pulmonary and hepatic injuries (5,6). For example, Xiong *et al* (7) showed that Sev could attenuate ventilator-induced lung injury by suppressing pulmonary inflammation in mice. In addition, Li *et al* (8) found that Sev preconditioning ameliorated spinal cord I/R-induced neuronal injury by inhibiting microglial MMP-9 expression in rats. Previous research has also demonstrated the neuroprotective effect of Sev on severe cerebral ischemia in rats (9-11). For example, pretreatment with Sev improved spatial learning and memory impairment after cerebral I/R injury in rats (12). However, the underlying mechanisms responsible for the protective effects of Sev against cerebral I/R injury are still elusive.

The present study determined the protective effects of Sev in a model of neuronal HT22 cell with oxygen-glucose deprivation/reperfusion (OGD/R), and investigate the involvement of the PI3K/AKT/GSK3 β signaling pathway in molecular mechanisms. The results of the present study suggest that Sev may be an improved anesthetic option for patients with cerebral I/R injury.

Materials and methods

Cell culture. The mouse hippocampal neuronal HT22 cell line was purchased from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal

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bovine serum (FBS; both from Sigma-Aldrich; Merck KGaA) with 4.5 g/l glucose, 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% CO₂ at 37°C. The medium was replaced every 2 days.

Induction of OGD/R injury. The OGD/R model was built as previously described (13). Briefly, the HT22 cells in 6-well plates (1x10⁶ cells/well; Costar 3506, Corning Life Sciences) were cultured under hypoxic conditions for 6 h in glucose-free DMEM in an atmosphere of 5% CO₂, 94% N₂ and 1% O₂ at 37°C. Subsequently, the medium was discarded and DMEM containing 4.5 g/l glucose was added, followed by culture under normoxic conditions in a humidified 5% CO₂ incubator at 37°C for 24 h. Cells without OGD/R treatment were used as a control.

Exposure to Sev. Cells in 6-well plates (1x10⁶ cells/well) were exposed to Sev in the carrier gas as previously described (14). Briefly, different concentrations of Sev (0, 4.1 or 8%) were delivered by a Sev vaporizer sustained in 21% O₂. The control gas was defined as 21% O₂. Cells in 6-well plates (1x10⁶ cells/well) were randomly placed in sealed plastic chambers and exposed to Sev or the control gas at 4 l/min for 6 h at 37°C, followed by induction of OGD/R injury. The concentration of Sev was monitored by an agent analyzer (AbbVie Inc.)

Drug treatment. The PI3K inhibitor, wortmannin (15) was obtained from Merck KGaA and dissolved in dimethyl sulfoxide (DMSO). HT22 cells in 6-well plates (1x10⁶ cells/well) were pretreated with 1 μ M wortmannin for 24 h, and then were exposed to 4.1% Sev for 6 h at 37°C, followed by OGD/R stimulation. The dose of wortmannin (1 μ M) was chosen as previously described (16).

Cell viability assay. At 24 h after sevo treatment, HT22 cells in 96 microplates (5x10⁴ cells/well) were subjected to OGD/R, and then the cell viability of HT22 cells was measured with the Cell Counting Kit-8 assay (CCK-8; Beyotime Institute of Biotechnology) according to the manufacturers protocols. A total of 10 μ l CCK-8 reagent in DMEM was added to the cells and incubated for another 2 h at 37°C. Absorbances at 450 nm were measured using a microplate reader (Bio-Rad Laboratories, Inc).

Lactate dehydrogenase (LDH) assay. At 24 h after sevo treatment, HT22 cells were subjected to OGD/R, and LDH release in the HT22 cells was measured using Pierce LDH Cytotoxicity Assay Kit (cat. no. 88953; Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The OD values were measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc).

Cell apoptosis assay. The apoptotic rate of HT22 cells was evaluated by Annexin V-FITC and PI apoptosis detection kit I (BD Pharmingen; BD Biosciences), following the manufacturer's protocol. At 24 h after sevo treatment, HT22 cells were subjected to OGD/R, and then cells were collected, washed with phosphate-buffered saline (PBS) and resuspended in binding buffer, and double-stained with Annexin V-FITC

and PI for 10 min at room temperature in the dark. After incubation for 15 min at room temperature in the dark, cell apoptosis was analyzed by FACScan flow cytometer (FCM; Beckman Coulter, Inc.) and CellQuest software version 3.3 (BD Biosciences). The apoptosis rate was the sum of the Q2 and Q3 quadrants of the flow cytometry images.

Caspase-3 activity. The caspase-3 activity of HT22 cells was measured after exposure to the various treatments using the Caspase-3 Assay kit (cat. no. C10427; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The optical density was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Detection of reactive oxygen species (ROS), malonaldehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx). ROS production in HT22 cells was measured using DCFH-DA (Invitrogen; Thermo Fisher Scientific, Inc.) as previously reported (17). At 24 h after sevo treatment, HT22 cells were subjected to OGD/R, and HT22 cells (1x10⁶ cells/well) were seeded in a 6-well plate for 24 h, and then stained with 20 μ mol/l DCFH at 37°C for 30 min, and then cell images were captured using a fluorescence microscope (Olympus Corporation) and analyzed by ImageJ software (version 1.46; National Institutes of Health).

After the designated treatment of interest, the HT22 cells were lysed using lysis buffer (Beyotime Institute of Biotechnology), following by lysate collection for the detection of SOD, MDA and GPx. The MDA content was measured by thiobarbituric acid method using a Lipid Peroxidation MDA assay kit (cat. no. S0131S), the SOD activity was detected by water-soluble tetrazolium salt (WST-8) method using a SOD assay kit (cat. no. S0103) and the activity of GPx was evaluated using a Cellular GPx assay kit (cat. no. S0056), all purchased from Beyotime Institute of Biotechnology, performed according to the manufacturer's instructions.

Western blot analysis. Western blot was performed as previously described (18). Briefly, total protein was isolated from HT22 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). The concentrations of total cellular protein were quantitated using BCA assay (Pierce; Thermo Fisher Scientific, Inc.). The protein lysates (40 μ g) were analyzed on an 8% SDS-PAGE gel and transferred onto PVDF membranes (EMD Millipore), followed blocking with 5% skim milk at 4°C overnight. The membranes were probed with the following appropriate primary antibodies: Phosphorylated (phosp)-AKT (Ser473; dilution 1:500; cat. no. 4060), AKT (dilution 1:1,000; cat. no. 4691), GSK3 β (dilution 1:1,000; cat. no. 5676), phosp-GSK3 β (Ser9; dilution 1:1,000; cat. no. 9327) and β -actin (dilution 1:2,000; cat. no. 4970) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated (HRP) antibodies (dilution 1:6,000; cat. no. 45262; Cell Signaling Technology, Inc.) for 1 h at room temperature. All antibodies were obtained from Cell Signaling Technology, Inc. β -actin served as an internal control. The protein bands were visualized using ECL detection reagent (Cytiva). The intensity of protein fragments was quantified with Bio-Rad Laboratories Quantity One software (version 3.0; Bio-Rad Laboratories, Inc.).

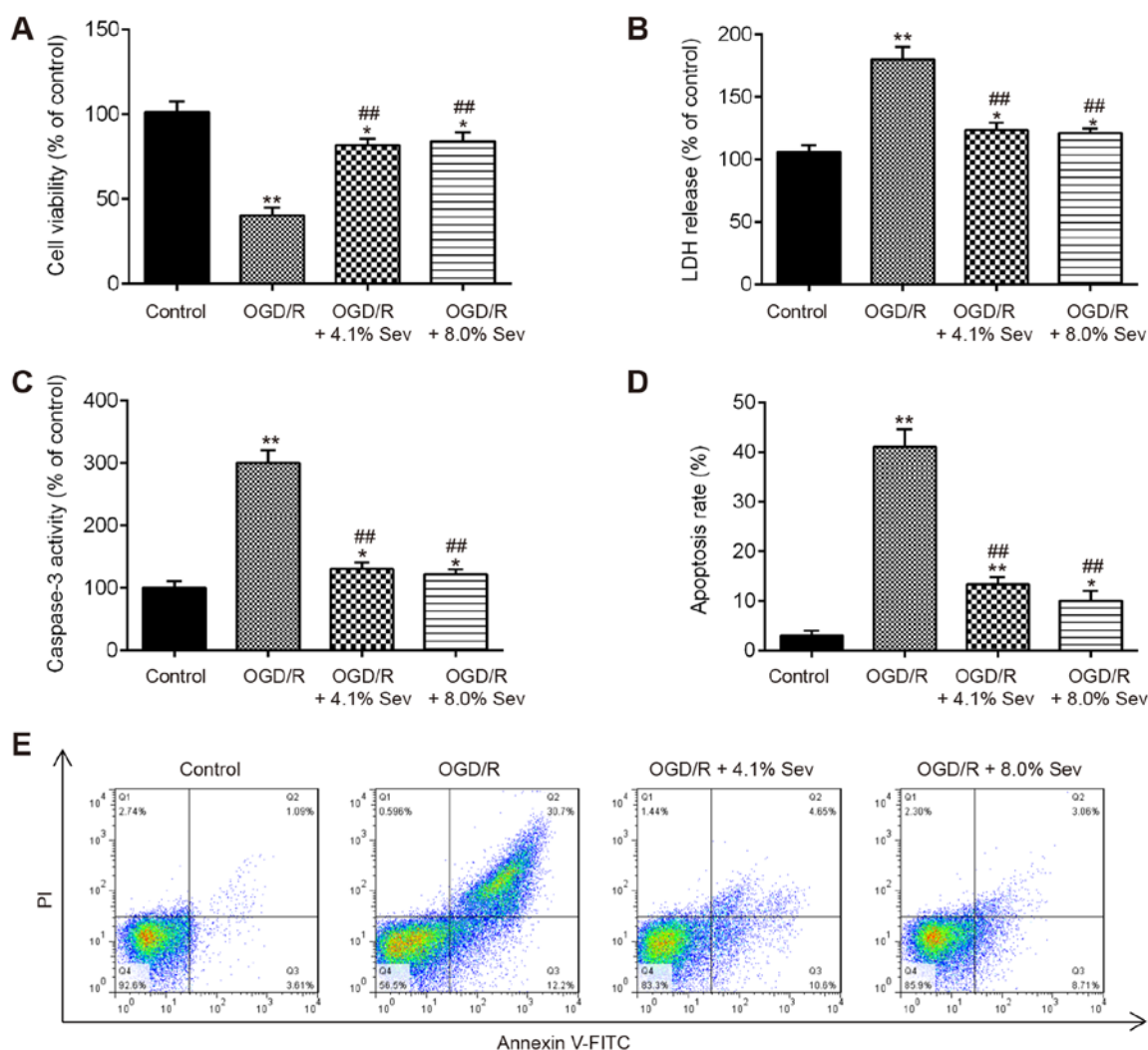


Figure 1. Effect of Sev on OGD/R-induced cell injury in mouse hippocampal neuronal HT22 cells. The HT22 cells were pretreated with Sev (4.1 or 8.0%), followed by OGD/R stimulation. (A) Cell viability of HT22 cells was determined using Cell Counting Kit-8. (B) LDH release was measured using the Pierce LDH Cytotoxicity assay kit. (C) Caspase-3 activity was measured using the Caspase-3 assay kit. (D and E) Apoptosis was measured by flow cytometry. Data are presented as the mean \pm standard deviation of three individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group. ## $P < 0.01$ vs. OGD/R group. LDH, lactate dehydrogenase; OGD/R, oxygen-glucose deprivation/reperfusion; Sev, sevoflurane.

Statistical analysis. Statistical analysis was performed by SPSS software version 14.0 (SPSS, Inc.). All data were presented as the mean \pm standard deviation. The comparisons among data were calculated by one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Sev ameliorates OGD/R-induced injury in HT22 cells. To evaluate the protective effects of Sev on the injury induced by OGD/R, HT22 cells were exposed to Sev (0, 4.1 or 8%) for 6 h, followed by induction of OGD/R injury. CCK-8 assay showed that cell viability was significantly decreased in OGD/R-induced cells, compared with that in the control group (Fig. 1A). However, Sev treatment (4.1 and 8%) significantly improved the viability of HT22 cells receiving OGD/R stimulation (Fig. 1A). The LDH assay showed that Sev treatment (4.1 and 8%) markedly decreased LDH leakage in OGD/R-induced HT22 cells, compared with the OGD/R group

(Fig. 1B). Given that apoptosis is a key event in the pathogenesis of cerebral I/R injury (19), Sev treatment was investigated for its potential to affect the apoptosis in OGD/R-induced HT22 cells. As shown in Fig. 1C, OGD/R significantly increased the caspase-3 activity, compared with the control group, whereas Sev treatment attenuated the promoting effect of OGD/R on the caspase-3 activity. Moreover, it was observed that OGD/R significantly promoted the apoptosis portion of HT22 cells, whereas Sev treatment decreased the apoptosis portion of HT22 cells (Fig. 1D and E). These data indicated that Sev treatment ameliorates OGD/R-induced cell injury and apoptosis in HT22 cells.

Sev inhibits OGD/R-induced oxidative stress in HT22 cells. Oxidative stress is another key factor in brain damage after cerebral I/R (20). Previous studies suggested that cerebral I/R could cause oxidative stress and then lead to over-production of ROS. Excessive ROS promote cell death and result in brain damage. Malondialdehyde (MDA) is the parameter of oxidative stress and is the lipid peroxidation product that is

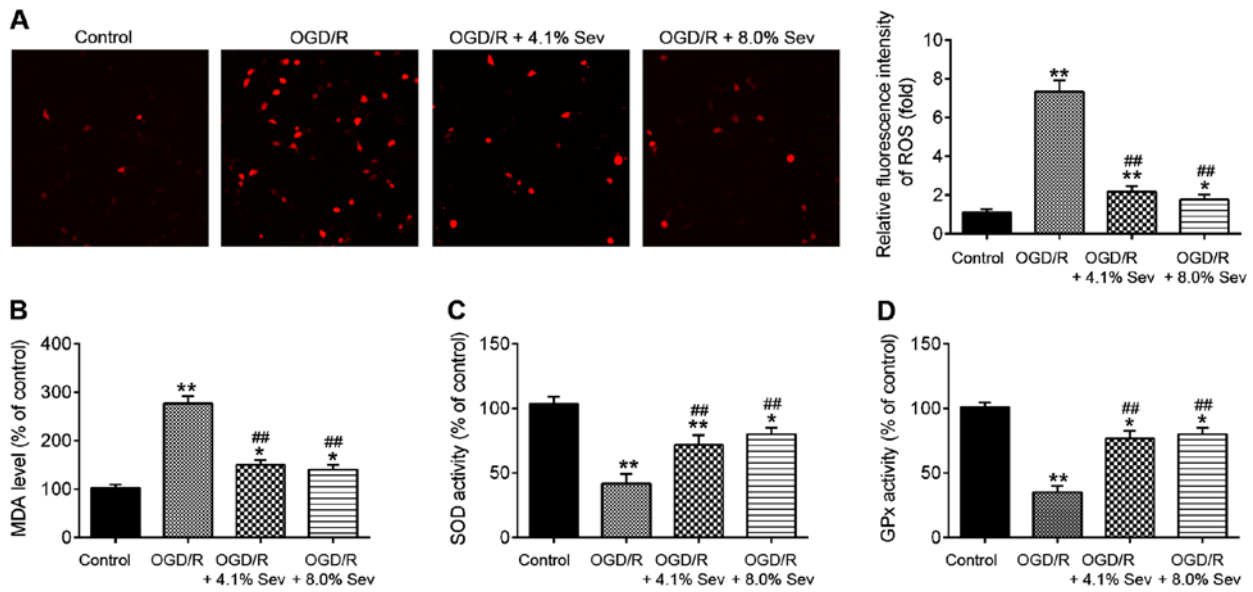


Figure 2. Effect of Sev on OGD/R-induced oxidative stress in neuronal cells. The mouse hippocampal neuronal HT22 cells were pretreated with Sev (4.1 or 8.0%), followed by OGD/R stimulation. (A) ROS were detected using DCFH-DA assay (x200 magnification). (B) MDA was detected with the Lipid Peroxidation MDA assay kit. (C) SOD activity was measured using the Total Superoxide Dismutase assay kit. (D) The activity of GPx was evaluated with the Cellular Glutathione Peroxidase Assay kit. Data are presented as the mean \pm standard deviation of three individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group. ## $P < 0.01$ vs. OGD/R group. GPx, glutathione peroxidase; MDA, malonaldehyde; OGD/R, oxygen-glucose deprivation/reperfusion; ROS, reactive oxygen species; Sev, sevoflurane; SOD, superoxide dismutase.

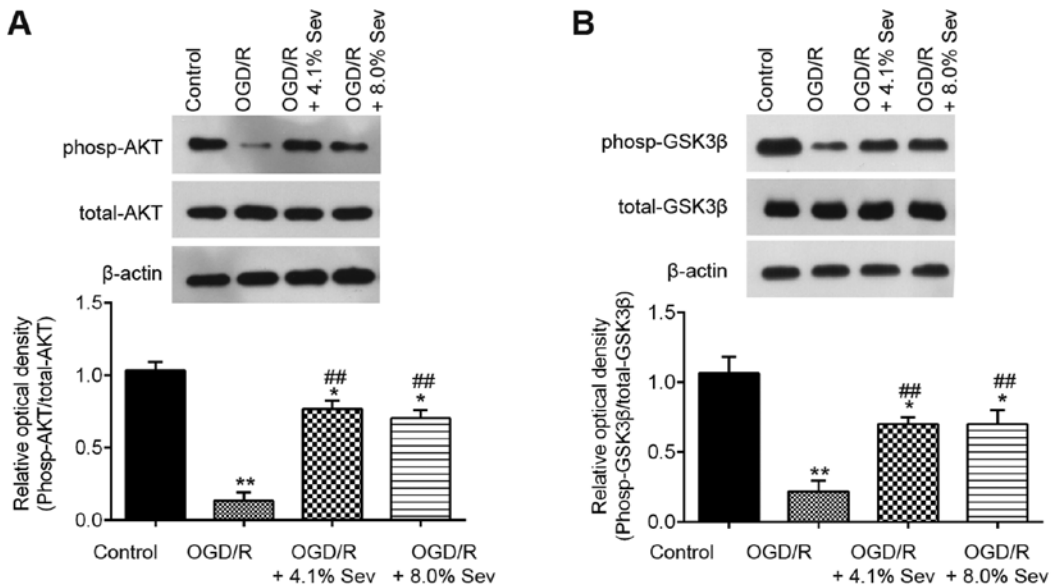


Figure 3. Sev induces the activation of PI3K/AKT/GSK3 β signaling pathway. The mouse hippocampal neuronal HT22 cells were pretreated with Sev (4.1 or 8.0%), followed by OGD/R stimulation. (A and B) Western blot analysis was used to detect the phospho-AKT, AKT, phospho-GSK3 β and GSK3 β expression levels. β -actin was used as an internal control for protein loading. Data are presented as the mean \pm standard deviation of three individual experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group. ## $P < 0.01$ vs. OGD/R group. OGD/R, oxygen-glucose deprivation/reperfusion; Sev, sevoflurane; GSK3 β , glycogen synthase kinase-3 β ; phosp, phosphorylated.

used to determine the level of damage of cell membrane lipid by ROS (21). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are antioxidant enzymes to protect cells from oxidative damage (22). Therefore, the detection of MDA, GSH-Px levels, and SOD activity reflect the degree of oxidative damage in cells and tissues. To examine whether Sev ameliorates OGD/R-induced cell injury by regulating oxidative stress, the markers of oxidative stress, namely ROS, MDA, SOD and

GPx, were detected after OGD/R injury and Sev treatment. As shown in Fig. 2A and B, ROS and MDA production levels were significantly increased in OGD/R-treated HT22 cells compared with those in the control group. However, the increased production of ROS and MDA in OGD/R-induced HT22 cells was decreased by Sev treatment (Fig. 2A and B). In addition, it was observed that OGD/R-induced a significant decrease in the activity of SOD and GPx in HT22 cells,

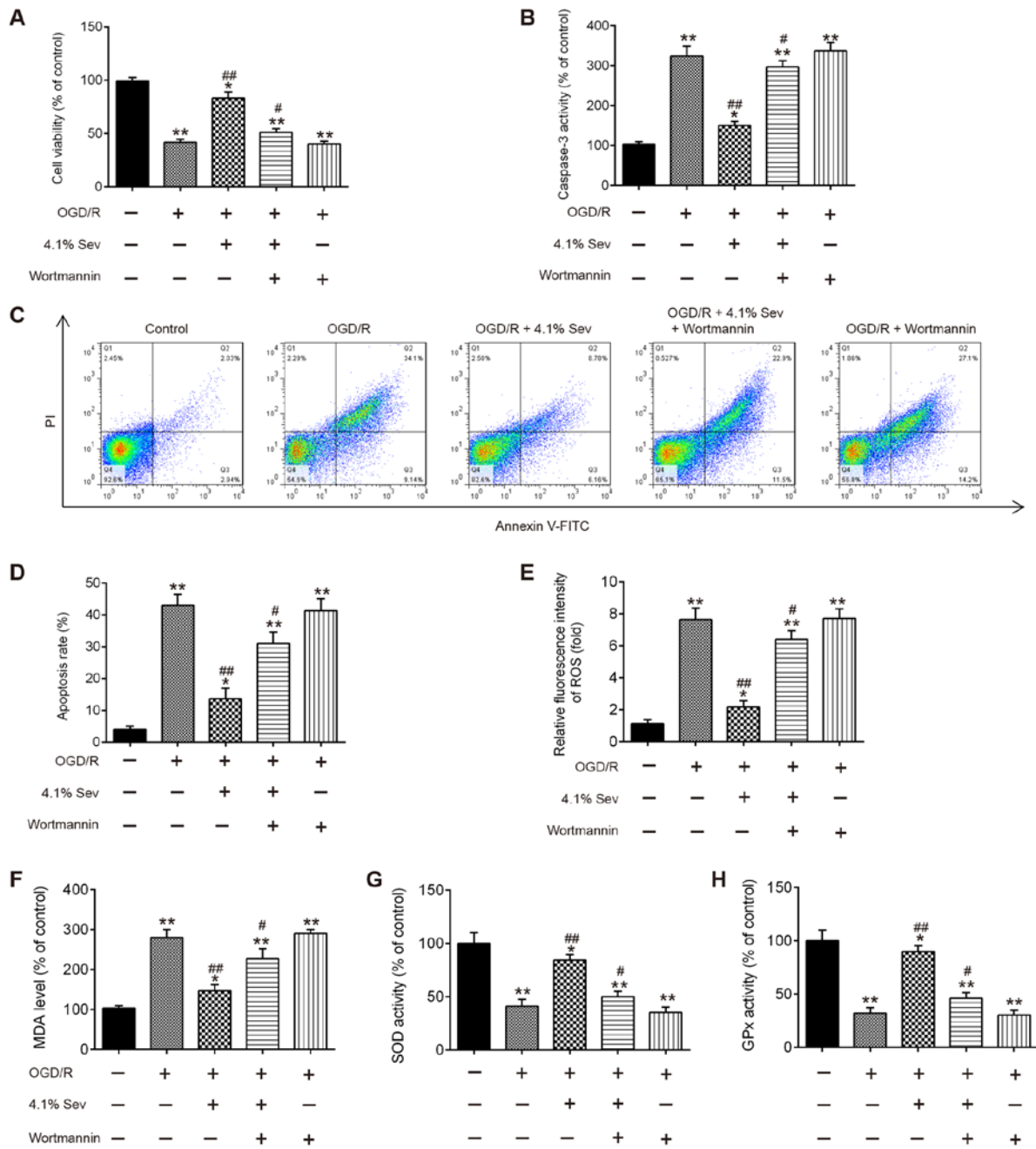


Figure 4. Inhibition of PI3K by wortmannin reverses the protective effect of Sev on cell injury in OGD/R-induced neuronal cells. The HT22 cells were pretreated with wortmannin (10 μ M) for 24 h, followed by OGD/R stimulation and then exposure to Sev (4.1%) for 6 h. (A) Cell viability was detected using Cell Counting Kit-8. (B) Caspase-3 activity was assessed using the Caspase-3 assay kit. (C and D) Apoptosis was measured by flow cytometry. (E) ROS were measured using DCFH-DA staining assay. (F) MDA content was measured using the Lipid Peroxidation MDA assay kit. (G) SOD activity was evaluated using the Total SOD assay kit. (H) The activity of GPx was assessed using the Cellular GPx assay kit. Data are presented as the mean \pm standard deviation of three individual experiments. * $P < 0.05$, ** $P < 0.05$ and *** $P < 0.01$ vs. control group. ## $P < 0.01$ vs. OGD/R group. GPx, glutathione peroxidase; MDA, malonaldehyde; OGD/R, oxygen-glucose deprivation/reperfusion; ROS, reactive oxygen species; Sev, sevoflurane; SOD, superoxide dismutase.

whereas Sev attenuated the inhibitory effects of OGD/R on the activity of SOD and GPx (Fig. 2C and D). All data suggest that Sev treatment may improve OGD/R-induced injury by suppressing oxidative stress in HT22 cells.

Sev induces phosphorylation of GSK3 β via the PI3K/AKT pathway. It has been previously reported that Sev provides neuroprotection in adult animals via the PI3K/Akt/GSK3 β signaling pathway, which has a key role in cerebral I/R injury (23,24). To explore the potential molecular mechanisms of Sev

against OGD/R-induced cell injury in HT22 cells, western blotting analysis was performed to detect the phosph-AKT, AKT, phosph-GSK3 β and GSK3 β levels in OGD/R-induced cells. As shown in Fig. 3A, phosph-AKT expression was markedly downregulated in OGD/R-induced cells compared with that in control cells, whereas Sev treatment attenuated the inhibitory effect of OGD/R on the expression of phosph-AKT. Furthermore, Sev treatment ameliorated the decrease in phosph-GSK3 β expression induced by OGD/R in HT22 cells (Fig. 3B). These results indicated that Sev could

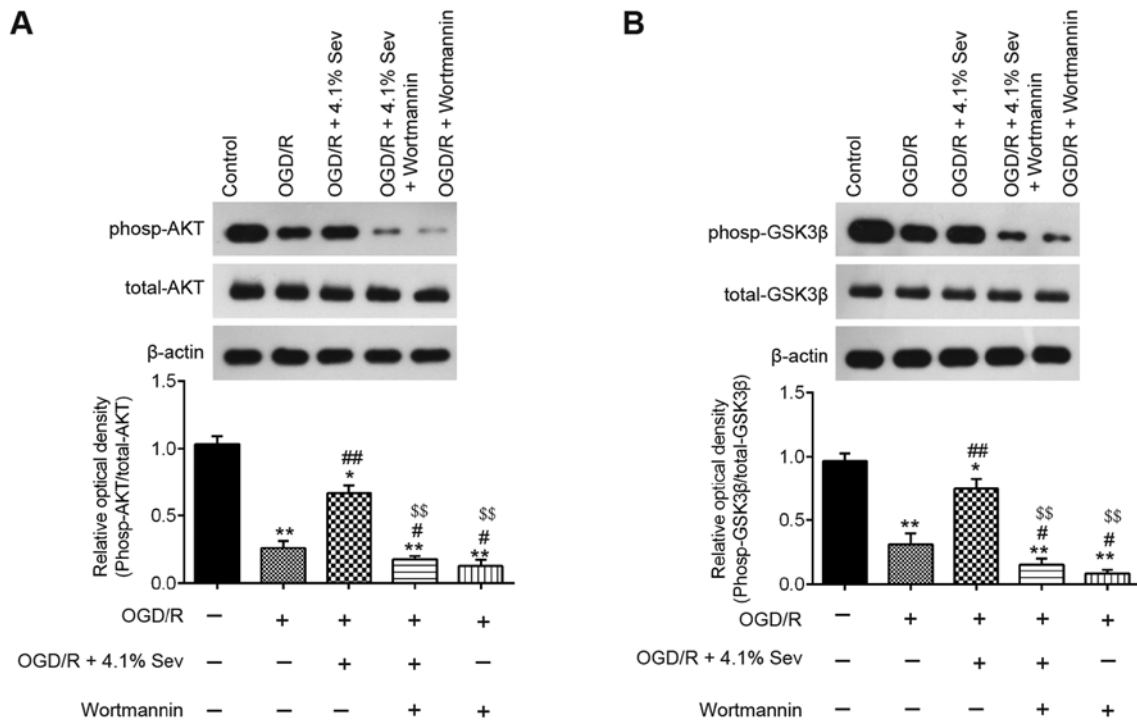


Figure 5. Sev-induced PI3K/AKT/GSK3 β signaling pathway is blocked by the inhibition of PI3K in OGD/R-induced cells. The HT22 cells were pretreated with wortmannin (10 μ M) for 24 h, followed by OGD/R stimulation and then exposure to Sev (4.1%) for 6 h. (A and B) The phospho-AKT, AKT, phospho-GSK3 β and GSK3 β expression levels were detected using western blot analysis. β -actin was used as an internal control for protein loading. Data are presented as the mean \pm standard deviation of three individual experiments. *P<0.05 and **P<0.01 vs. control group. #P<0.05, ##P<0.01 vs. OGD/R group. \$\$P<0.01 vs. OGD/R + Sev-treated group. GSK3 β , glycogen synthase kinase-3 β ; OGD/R, oxygen-glucose deprivation/reperfusion; Sev, sevoflurane.

reactivate the PI3K/AKT/GSK3 β pathway in HT22 cells after OGD/R induction.

PI3K kinase inhibitor wortmannin inhibits the protective effect of Sev on OGD/R-induced cell injury. To investigate the effects of the PI3K/AKT/GSK3 β signaling pathway on the neuroprotective effect exerted by Sev, wortmannin, a selective PI3K inhibitor, was added to HT22 cells in combination with Sev for 30 min before subjecting them to OGD/R at a concentration of 10 μ M. As shown in Fig. 4A, Sev treatment (4.1%) markedly promoted the cell viability in OGD/R-induced HT22 cells. However, the protection of Sev was significantly alleviated by the presence of 10 μ M wortmannin, whereas wortmannin alone did not influence the cell viability of HT22 cells compared with OGD/R-induced cells alone (Fig. 4A). Additionally, the results showed that pretreatment with wortmannin partially reversed the inhibitory effect of Sev on caspase-3 activity and apoptosis in OGD/R-induced cells (Fig. 4B-D). Moreover, blockade of PI3K by wortmannin also markedly reversed the protective effect of Sev on oxidative stress induced by OGD/R (Fig. 4E-H). All these data suggested that Sev may suppress OGD/R-induced apoptosis and oxidative stress through the PI3K/AKT/GSK3 β signaling pathway.

Sev-reactivated PI3K/AKT/GSK3 β signaling pathway is inhibited by wortmannin in OGD/R-induced cells. To confirm whether Sev regulated the PI3K/AKT/GSK3 β signaling pathway in OGD/R-induced cell injury, phospho-AKT, AKT, phospho-GSK3 β and GSK3 β expression levels were detected in OGD/R-induced HT22 cells treated with Sev and pretreated

with wortmannin. As shown in Fig. 5A and B, Sev treatment significantly increased the levels of phospho-AKT and phospho-GSK3 β compared with OGD/R-induced cells alone, but these effects were inhibited by wortmannin treatment. These data indicate that Sev alleviates OGD/R-induced cell injury through reactivating the PI3K/AKT/GSK3 β signaling pathway.

Discussion

In the present study, the results demonstrated that Sev improves OGD/R-induced neuronal injury by suppressing cell apoptosis and oxidative stress. Moreover, data revealed that the PI3K/AKT/GSK3 β axis is involved in the protective effects of Sev. The findings of the current study suggested that Sev has the potential to protect neurons from cerebral ischemic injury.

Sev, a volatile anesthetic with minimal pungency, low solubility and low toxicity, is used widely in anesthetic practice (25), and has been documented to play a neuroprotective role following severe cerebral ischemia. For example, Canas *et al* (10) showed that Sev has a neuroprotective effect in an *in vitro* model of ischemia-reoxygenation. Wise-Faberowski *et al* (26) found that Sev can attenuate OGD-induced neuronal cell death. Ye *et al* (19) reported that delayed application of Sev after reperfusion provides neuroprotection by activating the PI3K/Akt pathway, and Wen *et al* (27) demonstrated the neuroprotection of Sev against I/R-induced brain injury through the inhibition of JNK3/caspase-3 by enhancing the Akt signaling pathway.

Together, the results of these previous studies make Sev an attractive agent for the preservation of neuronal function. In the present study, using an OGD/R model, Sev pretreatment was found to markedly increase cell viability and attenuate LDH leakage in HT22 cells following OGD/R. In addition, Sev inhibited OGD/R-induced apoptosis, as evidenced by the decrease in caspase-3 activity and cleaved-caspase-3 protein expression in the HT22 cells. These data suggested that Sev can efficiently attenuate OGD/R-induced neuronal cell injury and apoptosis.

It is well known that oxidative stress is an important factor in the pathogenesis of cerebral I/R injury, which is caused by increased production of ROS and decreased activity levels of scavenger enzymes, such as SOD and GPx (28). ROS can interfere with the regulation of gene expression, thus altering the relative expression levels of signaling proteins, influencing intracellular signaling cascades (29). Under pathological conditions, such as cerebral ischemia, the aberrant production of ROS can result in the oxidation of cellular components, leading to cell damage (30). Hence, investigating whether Sev plays a role in ROS production after cerebral I/R injury was a focus of the present study. Sev was found to decrease the production of ROS and MDA, and to elevate the SOD and GPx activity induced by OGD/R in HT22 cells, suggesting that Sev protects neuronal cells from OGD/R injury by inhibiting oxidative stress.

PI3K/AKT, an anti-apoptotic and pro-survival kinase signaling cascade, plays an important role in I/R induced brain injury (31-33). Activation of AKT induces the phosphorylation of GSK3 β at serine 9 (34,35). In fact, the PI3K/AKT/GSK3 β signaling pathway plays a key role in modulating cellular survival/death after hypoxia and I/R (36-38). Recently, Gerace *et al* (39) indicated that inhibition of AKT/GSK3 β signaling pathway by the PI3K inhibitor LY294002 or two GSK3 β inhibitors exerts a neuroprotective effect against cerebral I/R injury induced by OGD/R (39). In addition, suppression of PI3K/AKT/GSK3 β signaling pathway by the PI3K inhibitor LY294002 and GSK3 β inhibitor LiCl exhibited neuroprotective properties against cerebral ischaemia-induced apoptosis (40). Therefore, we hypothesize that the neuroprotective effect of Sev on cerebral I/R injury occurs through the modulation of the PI3K/AKT/GSK3 β signaling pathway. Consistent with the aforementioned studies, the activation of the PI3K/Akt pathway was also demonstrated to mediate the protective effect of Sev on OGD/R-induced cell injury in the present study. However, it is worthy of note that wortmannin, a selective PI3K inhibitor, abolished the neuroprotective effect of Sev by suppressing the phosphorylation of AKT and GSK3 β proteins. Collectively, these results suggested that Sev improves OGD/R-induced neuronal injury by activating the PI3K/AKT/GSK3 β signaling pathway.

In summary, the results of the present study demonstrated that Sev alleviates OGD/R-induced neuronal injury through regulating the PI3K/AKT/GSK3 β signaling pathway. These findings suggest that the application of Sev may help to protect neurons against cerebral I/R injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QY, HD, YJ and YZ performed the experiments, analyzed the data and wrote the study. JZ conceived and designed the study and contributed reagents to the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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