Sevoflurane preconditioning reduces brain inflammation after experimental stroke by enhancing glycogen synthesis kinase-3 β/ nuclear factor erythroid 2-related factor - dependent microglial M2 polarization

Min Cai
Department of Psychiatry, Xijing Hospital, Forth Military Medical University
Sisi Sun
Medical Department of Xi'an Emergency Center
Shiquan Wang
Forth Military Medical University
Beibei Dong
General Hospital of Tianjin Medical University
Li Tian
Fourth Military Medical University
Hailong Dong
Fourth Military Medical University
Wugang Hou (gangwuhou@163.com)
Fourth Military Medical University https://orcid.org/0000-0003-0027-0150

Research

Keywords: sevoflurane preconditioning, ischemic stroke, glycogen synthesis kinase-3β, nuclear factor erythroid 2-related factor, M1/M2 microglia, inflammatory process

Posted Date: December 13th, 2019

DOI: https://doi.org/10.21203/rs.2.18794/v1

Abstract

Background: Sevoflurane preconditioning (SPC) results in cerebral ischemic tolerance; however, the mechanism remains unclear. In current study, we aimed to assess the M1/M2 shift in the brain induced by SPC and whether glycogen synthesis kinase-3β (GSK-3β)-regulated nuclear factor erythroid 2-related factor (Nrf2) activation was involved in the M2 polarization mediated by SPC.

Methods: Mouse primary microglia with SPC were challenged by oxygen-glucose deprivation (OGD) or lipopolysaccharide (LPS), and mice with SPC were subjected to middle cerebral artery occlusion (MCAO). Then, the mRNA and protein levels of proinflammatory/anti-inflammatory factors were analysed. GSK-3β phosphorylation and Nrf2 nuclear translocation were measured. The mRNA and protein expression of proinflammatory/anti-inflammatory factors, neurological scores, infarct volume, cellular apoptosis, the proportion of M1/M2-positive cells, and the generation of super-oxidants were examined after SPC or GSK-3β inhibitor TDZD treatment with or without Nrf2 deficiency.

Results: SPC promoted M2 phenotype polarization both in vitro and in vivo . GSK-3β phosphorylation at Ser9 was increased after SPC. Both SPC and TDZD administration enhanced Nrf2 nuclear translocation, promoted M2 phenotype polarization and elicited a neuroprotective effect. Nrf2 deficiency abolished the promoted M2 polarization and ischemic tolerance induced by TDZD treatment. The reduced percentage of M1-positive cells and super-oxidants generation induced by SFC or TDZD was also reversed by Nrf2 knockdown.

Conclusions: Our results indicated that SPC exerts brain ischemic tolerance and enhances M2 polarization by GSK-3β-dependent Nrf2 activation, which provides a novel mechanism for SPC-induced neuroprotection.

Introduction

Cerebral ischemia/reperfusion (I/R) that occurs during cardio-cerebral surgery has serious adverse effects on patient prognosis[1]. Unfortunately, few therapies for the prevention and treatment of perioperative ischemic stroke have been clinically approved. Therefore, more therapeutic strategies for perioperative stroke are urgently needed. Sevoflurane preconditioning (SPC) results in tolerance against subsequent experimental cerebral I/R damage in vitro and in vivo[2–5]. However, the exact molecular and subcellular mechanisms underlying the neuroprotective property of this volatile anaesthetic are still unclear.

One of the most important pathophysiological features of I/R injury is the activation of the inflammatory response in the central nervous system (CNS)[6]. Although the exact mechanism is not completely understood, activation of microglia seems to be a characteristic process during I/R-induced excessive brain inflammation[7]. As cerebral immunocompetent resident cells, microglia have particular properties suitable for mediating cellular inflammatory responses during ischemia, which in turn reinforce the I/R injury[8–10]. Among these, the switching between the proinflammatory M1 and the anti-inflammatory M2 microglia phenotype is crucial in the regulation of inflammatory/anti-inflammatory gene expression and

in the determination of excessive brain damage[10–12]. Therefore, the M1/M2 shift-regulated inflammatory process could be a target of preconditioning approaches to produce protective effects against cerebral ischemic damage.

As a multifunctional serine/threonine kinase, glycogen synthase kinase-3β (GSK-3β) is highly active in resting cells and usually inhibits multiple downstream pathways. GSK-3β drives a cascade of signalling pathways, including inflammatory responses in the brain[13–15]. This enzyme participates in the production of proinflammatory factors, while pharmacological or genetic inhibition of this kinase could act as a molecular brake to limit the brain inflammatory response[15, 16]. Accumulating evidence has demonstrated the major role of GSK-3β inhibition in the prevention of neuronal death, including the brain ischemic tolerance induced by SPC[14, 17, 18]. However, to our knowledge, the underlying mechanism of GSK-3β that governs the anti-inflammatory processes caused by the M1/M2 shift and its effects on the neuroprotection produced by SPC are still unknown.

Furthermore, in addition to Kelch-like ECH-associated protein 1 (Keap1)-mediated Nrf2 degradation, GSK-3β has also been identified as a novel upstream regulator of Nrf2[19, 20]. GSK-3β could phosphorylate numerous Ser residues in the Neh6 domain of Nrf2, which overlap with an SCF/β-TrCP destruction motif to promote Keap1-independent Nrf2 degradation[21, 22]. Nrf2 acts as a “master regulator” in response to oxidative electrophilic stress and chemical insults and is also a major modulator factor associated with the shift of the M1/M2 phenotype in response to cerebral I/R injury[12, 23, 24]. Recent studies have shown that GSK-3β inhibition-induced Nrf2 activation plays a crucial role in protecting organs from I/R injury[18, 21, 25]. However, whether this signalling pathway involves the promotion of microglia polarization towards the M2 phenotype and the reduction of neuroinflammation induced by SPC is still unclear.

In the present study, we used oxygen-glucose deprivation (OGD)/lipopolysaccharide (LPS) stimulation in primary mouse microglia culture and a transient focal cerebral I/R model in mice to investigate the role of GSK-3β/Nrf2-dependent microglia M2 polarization in the neuroprotection induced by SPC.

Materials And Methods

Animals

All animal-related procedures were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (Xi’an, China) and proceeded in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Moreover, the randomization and analgesia procedures were performed in compliance with the ARRIVE guidelines. A randomized number table was used for randomization. The analgesia protocol involved administration of meloxicam (i.p.) at 0.2 mg/kg body weight postoperatively and was followed by administration of 0.05 mg/kg body weight for 3 consecutive days.

Male C57BL6j mice between 8 to 10 weeks old (25–30 g) were purchased from the Animal Laboratory of the Fourth Military Medical University, Xi'an, China. The mice were housed in a 12 h alternating light and dark cycle at 20–25 °C and 60% humidity with freely available water and food for at least 1 week prior to treatment or surgery. The sample size was based on our previous study and the formal statistical power analysis wasn't use to guide sample size of this study[3]. The number of animals used and their suffering were minimized in this study.

Experimental protocols

Experiment 1. Analysis of the mRNA expression of M1 marker genes (tumour necrosis factor-α, TNF-α; interleukin-1β, IL-1β; inducible nitric oxide synthase, iNOS) and M2 marker genes (CD-206, YM1/2, arginase-1) in vitro and in vivo. Two in vitro models were applied in this experiment: OGD and LPS stimulation. The primary cortical microglia received different treatments as follows: control, OGD and SPC + OGD or control, LPS and sevoflurane SPC + LPS. After different treatments were confirmed, the mRNA levels were measured. Additionally, mice were randomly divided into three groups: control, I/R, and SPC + I/R. Seven days or fourteen days after reperfusion, the expression of proinflammatory factors (TNF-α, IL-1β and iNOS) was analysed by enzyme-linked immunosorbent assays (ELISAs). Changes in M1 marker and M2 marker genes were also examined at 7 and 14 days after reperfusion, respectively.

Experiment 2. Examination of GSK-3β phosphorylation after SPC and determination of the role of GSK-3β in the neuroprotective effect and the M1/M2 shift induced by SPC. Mice were randomly allocated to three groups: control, I/R and Sevo + I/R. The phosphorylation at Ser9 and total protein expression of GSK-3β were evaluated by Western blots. GSK-3β phosphorylation was also observed by immunofluorescence staining. Moreover, the GSK-3β inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD, 1 mg/kg in 10% DMSO, i.p., 1.5 h following reperfusion) was administered based on a previously reported procedure[26]. Mice were randomly divided into five groups as follows: control, I/R and SPC (Sevo + I/R), I/R + vehicle, and TDZD + I/R. Three days after reperfusion, the neurological outcomes, infarct volume and mRNA levels of M1 marker and M2 marker genes were assessed. An additional cohort of mice was used to measure apoptotic cell death by terminal deoxynucleotidyl transferase deoxyuridine triphosphate-biotin nick-end labelling (TUNEL) staining at 72 h after reperfusion.

Experiment 3. Verification of the downstream GSK-3β that regulates the M2 phenotype polarization induced by SPC. Mice were randomly divided into the control, I/R, Sevo + I/R, I/R + vehicle and TDZD + I/R groups, and Nrf2 nuclear translocation was assessed by Western blot analysis. Nrf2 expression was also examined by immunofluorescence staining. Moreover, an adeno-associated virus (AAV) shRNA method was used to induce Nrf2 deficiency in this experiment (AAV-Nrf2). Mice were divided into the following groups: control, I/R, TDZD, TDZD + AAV-Nrf2, and TDZD + AAV-GFP (control of AAV-Nrf2). Seven days after reperfusion, the neurological disorder, infarct size and mRNA levels of M1 marker and M2 marker genes were assessed. An additional cohort of mice was used to measure apoptotic cell death by TUNEL staining at 72 h after reperfusion.

Experiment. Given the crucial role of Nrf2 in the regulation of antioxidant mechanisms, the dihydroethidium (DHE) oxidation staining method was also used to demonstrate the effect of the GSK-3β/Nrf2 signalling pathway in SPC. For further analysis of the effect of this pathway on the proportion of active M1 and M2 microglia in the ischemic penumbra induced by SPC, flow cytometry analysis was performed. The mice were divided into the following groups: control, I/R, Sevo + I/R, TDZD + I/R, TDZD + AAV-Nrf2 and TDZD + AAV-GFP. The two experiments mentioned above were performed according to previous studies.

Cell Culture of Mouse Primary Cortical Microglia

Culture of mouse primary cortical microglia was obtained from 24-h old C57BL/6 newborn pups[27]. Briefly, the entire brain of mouse were put in ice-pretreated D-hanks solution, then the meninges and other non-cortical tissue were separated and the whole cortex was harvested. The collection of brain cerebral cortex were digested with 0.25% trypsin (Invitrogen) at 37 °C for 7 min and followed by the supplement of DMEM/F12 (Invitrogen) in 10% FBS to stop the digestion. After this, the cortices were fully dissociated with pipettes. And, the cell suspension was subsequently filtered with a 70-μm-diameter mesh. Then the cells were transferred to a 75 cm^2 poly-lysine (PLL, Sigma)-coated flask and were incubated at 37 °C with 5% CO_2. About 50% of the culture media was replaced twice per week. After 10 days of culture, primary microglia were collected by shaking the flask for 2 h at 200 rpm and subsequently seeded onto PLL-pre-coated new plates for next experiments.

Sevoflurane Preconditioning in vitro

The procedure of sevoflurane preconditioning in vitro was based on previous publication[28]. Briefly, the primary microglia cells were placed in an incubator chamber (Billups-Rothenberg, San Diego, CA), which was flushed for 5 min with 2.5% sevoflurane in the carrier gas of 95% air-5% CO_2, and then sealed at 37 °C for 1 h. An anesthetic gas analyzer was used to monitor the concentration of sevoflurane in the chamber.

Oxygen and Glucose Deprivation

The OGD was performed as reported previously[4]. In brief, primary microglia cells were plated in DMEM with 10% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100/units) at 37 °C in 5% air. During OGD operation, the medium of culture was switched to serum- and glucose-free Dulbecco's Modified Eagle's Medium and placed in a modular incubator chamber subsequently, which was flushed with a mixture of 95% N_2 and 5% CO_2 at the rate of 3 L/min at room temperature for 30 min. Control cultures were incubated for the same period of time in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 4 h challenge, microglia cells were removed from the anaerobic chamber and the medium of culture was replaced by Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. The generation of reperfusion insult was confirmed by maintaining cells in a humidified 5% CO_2 incubator for further 24 h at 37 °C.

LPS Stimulation in vitro

The administration of LPS was performed according to the previously reported paradigm[29]. In brief, 24 h before LPS supplement, DMEM/10% FBS was replaced by DMEM/1% FBS. Then primary microglia cells were stimulated with LPS from Escherichia coli, serotype 055:B5 (Sigma, Buchs, Switzerland) in a concentration of 20 μg/mL in DMEM/1% FBS for 2 h. For control group, cells were only treated with PBS in DMEM/1% FBS instead of LPS.

Cell Viability Assay

The cell viability was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazol ium bromide (MTT) analyzing kit[30]. Shortly, microglia cells were cultured at 15×10^3 cells per well in 96-well tissue plates and subjected to various treatments described earlier. At the end of the culture period, cells were washed with PBS and MTT was added to each well for 4-h incubation at 37 °C in according to the manufacturer's instruction. Then, the medium was switched with dimethyl sulfoxide (Sigma-Aldrich). The optical density (OD) at 490 nm was measured by using a Universal Microplate Reader (Elx 800, Bio-TEK Instruments Inc., USA). Cell viability results were present as percentage changes of the control value in control group.

Assessment of Lactate Dehydrogenase (LDH) Release

To examine the cell cytotoxicity, LDH released from the injured cells into the culture medium after OGD/LPS stimulation was analyzed by LDH assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. LDH leakage results were shown as the percentage of the total LDH activity (LDH in the both medium and cells), according to the equation, LDH released (%) = (LDH activity in the medium/total LDH activity) × 100%. Cultures without OGD/LPS treatment (control group) were expressed as a basal LDH release.

Sevoflurane preconditioning in vivo

Sevoflurane preconditioning of the mice was achieved by inhalation of 97% O_2 containing 2.5 vol% sevoflurane for 1 h per day, 5 days continuously. The control group was treated with the same duration but was only allowed to inhale 97% oxygen. At 24 h washout period after the last treatment, mice were subject to MCAO surgery.

Arterial Blood Gas Measurement

Five additional mice in each group were used for arterial blood gas determination. About 0.2 ml blood of each mouse was taken respectively from femoral artery at the end of the last expose of sevoflurane or oxygen. Samples were analyzed immediately by using the OMNI Modular System (Rapidlab 1260, Bayer HealthCare, Uxbridge, United Kingdom).

Transient Middle Cerebral Artery Occlusion Model

Cerebral I/R injury was induced by transient middle cerebral artery model in mice as described previously. In brief, after an overnight fast, animals were anesthetized by 3% sevoflurane for induction and 2.5% for surgery. After the right carotid arteries were dissected out, an intraluminal 6 – 0 nylon monofilament with

a round tip was inserted from the right common carotid artery to the right middle cerebral artery. Following 1 h of transient occlusion, the filament was withdrawn to allow reperfusion. The temporal temperature was maintained at 37 ± 0.5 °C by a thermostatic blanket and a lamp. Sham-operated mice in control group were only subjected to a same surgical procedure without insert the suture.

The regional cerebral blood flow (rCBF) was quantitated by a laser Doppler flowmeter (PeriFlux 5000; Perimed AB, sweden) placed on the right side of skull's dorsal surface (caudal 2 mm and lateral 5 mm to bregma) before, during, and after the operation. Mice were excluded if their rCBF didn't fall below 20% of baseline during occlusion or recover over 80% during reperfusion. Moreover, if the whole MCAO procedure couldn't complete within 10 min, the mice were also discarded.

Neurobehavioral Evaluation and Infarct Size Assessment

Neurological behavioral outcome in the mice was assessed in according with the Garcia Score Scale by an observer blinded to the animal groups[31]. The data was expressed as median (interquartile rage).

The infarct volume was assessed by TTC staining (n = 8) as previously described. Briefly, the mice were decapitated after the last neurobehavioral test. The brain was rapidly removed and cooled in ice-cold saline for 5 min. Coronal sections (1 mm) were prepared, immersed in 2,3,5-triphenyltetrazolium chloride (1%; TTC, Sigma-Aldrich, St. Louis, MO) for 10 min and then fixed with 4% paraformaldehyde in 0.01 M PBS (pH 7.4) for 24 h. The brain slices were photographed using a digital camera (Canon IXUS 220HS). The infarct volume was calculated by Swanson's method to correct for edema: 100%× (contralateral hemisphere volume - nonlesioned ipsilateral hemisphere volume)/contralateral hemisphere volume[32].

TUNEL Staining for Apoptosis Assessment

Cellular apoptosis was evaluated 72 hours after reperfusion by TUNEL staining. By using the in-situ cell death detection kit (Roche, German), TUNEL staining was performed on paraffin-embedded sections in according to a standard protocol reported. The positive cells were acquired using a 40 × objective lens from areas in the ischemic penumbra, and the number of TUNEL positive cells was expressed as number per 100 br^2. The ischemic penumbra area was defined as the paradigm reported previously[33].

Evaluation of Inflammatory Factors

In order to measure the expression of inflammatory factors in infarct hemisphere after reperfusion, the penumbra tissue was homogenized in cooled sodium chloride after dissociated and weighted. The homogenate was centrifuged at 10000 g for 15 min and the supernatant was collected and frozen at -80 °C for the later detection. The content of protein in supernatant of each sample was calculated by BCA protein assay kit (Beyotime, Nantong, China).

ELISA assay kits were used to assess the content of inflammatory factors (TNF-α, IL-1β and iNOS) in strict accordance with the manufactory's protocols. All protein analysis kits used in this study were purchased from Nanjing Jianchen Bioengineering Institute.

RNA Isolation and Quantitative PCR

Total RNA was extracted from primary microglia or brain penumbra by using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Isolated RNA was reverse transcribed into cDNA with a cDNA synthesis kit (Invitrogen, USA) in strict accordance of the standard procedures. Quantitative PCR was confirmed by using synthesis primes and SYBR Green (Invitrogen, USA) with an IQ5 Detection System (Bio-Rad). The PCR cycling began with template denaturing at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, and 78 °C for 20 s.

The primers used in this study were list as Table 1.

Western-blot

At 30 min and 2 h after reperfusion, mice from each group (n = 5) were euthanized. The brain tissue corresponding to the ischemic penumbra was harvested as previously described. The tissue was homogenized in ice-cold RIPA lysis buffer containing 1% phenylmethanesulfonyl fluoride (Beyotime, Nantong, China). For the examination of Nrf2 nuclear translocation, nuclear protein was exacted with a Nuclear Extraction Kit (Pierce Biotechnology, USA). The concentration of protein was measured by the Bradford method with an available kit (Beyotime, Nantong, China). The primary antibodies were listed as following: anti-GSK-3 l and p-GSK-3i (Ser-9) (1: 1,000, respectively; Cell signaling technology), anti-Nrf2 (1:500; Abcam), anti-glyceral-dehyde-3-phosphate dehydrogenase (1:2,000; Abcam) or histone-3 (1:500; Signal way antibody). After the membranes were incubated with appropriated secondary antibodies, the blots were immersed in enhanced chemiluminescent regent and then exposed to enhanced chemiluminescent-Hyperfilm (Amersham Biosciences) to visualize the specific protein bands.

Immunofluorescence Staining

For immunofluorescence staining, brain sections were washed with phosphate buffered saline containing 1% Triton for 3 times, then were incubated with rat anti-neuronal nuclei antibody (1:1000, Millipore), rabbit anti-p-GSK-3 (Ser-9) antibody (1:100, Cell Signaling Technology) or rabbit anti-Nrf2 antibody (1:100, Abcam) for 24 h at 4 °C. After washed with PBS for 3 times, the sections were incubated with CY3-labeled goat anti-rabbit and FITC-labeled goat anti-rat secondary antibodies (1:1000 for both, Millipore) at room temperature for 2 h. Sections was incubated with the nuclei marker DAPI (1 ng/u, Sigma) for 5 min at room temperature. Fluorescent signals were determined by using a confocal fluorescence microscope (Flv100i, Olympus).

Construction and Transfection of Nrf2-shRNA

The Nrf2-shRNA (AAV9-Nfe2l2-RNAi, 2.97×10^{12} v.g./ml) and control AAV9-GFP were purchased from GeneChem Co., Ltd. (Shanghai, China; No. GIDV0166296). The target sequence was 5'-CGCTGAGTACTTCGAAATGTC-3'. AAV-Nrf2 or AAV-GFP transfection was confirmed by intracerebroventricular injection. The stereotaxic coordinate location of the lateral cerebral ventricle was 0.4 mm posterior to the bregma, 1.0 mm lateral to the midsagittal line, and 2.0 mm deep from the cranial surface. At 72 h after injection, the efficacy of the shRNA was determined by immunofluorescence

labelling. As shown in Supplementary Fig. S3, Nrf2-shRNA but not Nrf2-shRNAc reduced the number of Nrf2-positive cells compared with the control.

Dihydroethidium Oxidation Staining

After deep anaesthetization, the mice were decapitated, and the brains were quickly removed and placed onto a base mold surrounded with TBS Tissue Freezing medium. After being placed in dry ice for 15 min, the brains were cut on a cryostat into 20-μm-thick sections. Sections were then incubated with DHE working solution (10 mM, Beyotime) at 37 °C for 30 min and covered with 24-mm square glass coverslips.

The images of DHE oxidation were captured by confocal fluorescence microscopy (Flv100i, Olympus). The quantification of DHE-positive cells was performed in accordance with the method reported by Dugan et al.[34].

Statistical Analysis

GraphPad Prism for Windows (version 7.0) was used to conduct the statistical analyses. The neurological score was expressed as the median with the interquartile range and analysed by a nonparametric Kruskal-Wallis test followed by a Mann-Whitney U test with a Bonferroni correction. The other values are presented as the mean ± S.D. and were analysed by one-way ANOVA. The differences between the two groups were detected with Tukey's post hoc test. Two-tailed values of $P < 0.05$ were statistically significant.

Results

SPC Promoted Mouse Primary Microglia Polarization into the Anti-Inflammatory M2 Phenotype and Elicited A Protective Effect against OGD

As illustrated in Fig. 1A left panel, OGD increased the lactate dehydrogenase (LDH) release to 496.4 (14.91%) compared to that of the control group ($P < 0.0001$), and SPC showed a significant effect on the prevention of increased LDH levels induced by OGD [357.8 (39.68%) in the Sevo + OGD group, $P = 0.0046$]. Compared with that in the control group, the cell viability in the OGD group was significantly reduced. SPC restored the cell viability [figure 1A, right panel, 36.42 (1.66%) in the OGD group vs. 74.37 (3.33%) in the Sevo + OGD group, $P = 0.0037$].

As shown in Fig. 1B, the proinflammatory factor (TNF-α, IL-1β, iNOS) mRNA levels in the OGD group were higher than those in the control group. When the mouse primary microglia cells were pre-treated with sevoflurane, the mRNA expression was decreased compared with that of the OGD group ($P = 0.0023$, < 0.001, 0.0073). The mRNA changes of anti-inflammatory mediators are presented in Fig. 1C. Treatment with OGD elevated the CD206, YM1/2 and arginase-1 mRNA levels compared with those in the control group ($P < 0.001$), while SPC prevented this increase in mRNA expression ($P = 0.0019$, < 0.001, and 0.007, respectively).

SPC Promoted Mouse Primary Microglial Polarization into the Anti-Inflammatory M2 Phenotype and Protected Cells against LPS-Induced Injury

LPS stimulation was also performed in this study to further determine the effect of SPC on microglia polarized to the M2 phenotype (Fig. 1D-F). As shown in Fig. 1D, the microglial culture was severely damaged by LPS stimulation, which was demonstrated by the increased LDH release and decreased MTT levels in the LPS group ($P < 0.001$). This toxicity was reduced when the microglial cells were pre-treated with sevoflurane ($P = 0.0023$ for LDH, $P = 0.022$ for MTT).

The LPS treatment significantly up-regulated the mRNA levels of M1 marker genes (TNF-α, IL-1β, and iNOS), while SPC exerted a significant effect on the inhibition of LPS-induced elevation of these proinflammatory genes ($P = 0.0033$, < 0.001, and 0.0015, respectively). In addition, SPC treatment improved the mRNA levels of M2 marker genes (CD206: $P = 0.0057$, YM1/2: $P = 0.0056$, arginase-1: $P = 0.0037$).

SPC Reduced Proinflammatory Factor Expression and Enhanced M2 Microglia/Macrophage Polarization in The Ischemic Hemisphere of The Mice at 7 and 14 Days After Reperfusion.

To demonstrate the correlation of SPC and M2 polarization in vivo, we measured the mRNA levels of proinflammatory and anti-inflammatory factors at 7 (Fig. 2A-B) and 14 (Fig. 2C-D) days after reperfusion in a transient MCAO model.

Changes in physiologic parameters at the end of the preconditioning operation and various intervals of I/R are summarized in Supplementary Tab. S1. No significant differences in the pH value, temporal temperature, or partial pressure of carbon dioxide (PCO_2) were detected among the groups. As shown in in Supplementary Fig. S1, SPC did not alter the regional cerebral blood flow.

As presented in Fig. 2A, the TNF-α, IL-1β and iNOS mRNA levels were increased at 7 days after I/R, while this elevation was reversed by pretreatment with 5 days of sevoflurane ($P = 0.0202$, 0.006, and < 0.001, respectively). As shown in Fig. 2B, I/R did not alter the mRNA expression of anti-inflammatory mediators (CD206: $P = 0.6507$, YM1/2: $P > 0.9999$, arginase-1: $P = 0.7854$). However, the mRNA levels of the anti-inflammatory mediators in the SPC-treated group were significantly increased compared with those of the I/R group ($P = 0.0006$, $P = 0.0013$, $P = 0.0012$).

Consistent with the results at 7 days after reperfusion, the mRNA expression of these proinflammatory factors was increased in the infarct penumbra at 14 days after reperfusion (Fig. 2C). However, the increased mRNA levels of TNF-α, IL-1β and iNOS were also prevented by SPC at this time-point ($P = 0.0006$, 0.0013, and 0.0012, respectively). As shown in Fig. 2D, no significant change in the mRNA levels of M2 marker genes was detected between the control and I/R groups (control vs. I/R: $P = 0.6081$, 0.2765, 0.1144), while SPC significantly increased the mRNA expression of these anti-inflammatory factors (I/R vs. Sevo + I/R: $P < 0.001$, $= 0.002$, < 0.001).

Sevoflurane Preconditioning Increased the Phosphorylation of GSK-3β, and Supplementation With A GSK-3β Inhibitor Reduced Cerebral I/R Injury

As shown in Fig. 3A, the GSK-3β phosphorylation was determined by Western blot analysis and immunofluorescence staining. The abundance of phosphorylated GSK-3β in the I/R groups was reduced compared with that of the control group (P = 0.0218), but this reduction was ameliorated in the SPC group (Sevo + I/R vs. I/R, P = 0.0206). Neither I/R nor Sevo + I/R treatment affected the total GSK-3β protein expression. As expressed in the right panel of Fig. 3A, the results of double immunofluorescence staining demonstrated that increased GSK-3 phosphorylation produced by SPC was mainly colocalized with neurons.

To further verify the role of GSK-3β in I/R tolerance induced by SPC, we used a GSK-3β inhibitor, TDZD, in this study. As shown in Fig. 3B, SPC significantly improved the neurobehavioral outcome, as judged by the neurological score, at 1 day [10.75, (9.00, 12.00)], 2 days [10.00 (7.00, 11.00)] and 3 days [9.50, (7.00, 11.00)] in the Sevo + I/R group compared with the I/R group at parallel time-points (P = 0.0031, 0.0121, and 0.0008, respectively, for each comparison). Consistent with the improvement in neurological outcome, the infarct volume in the Sevo + I/R group was smaller than that in the I/R group [figure 3C, 30.5 (2.9%) vs. 43.1 (3.1), P = 0.0142). Following the administration of TDZD, mice that underwent the MCAO surgery showed better neurobehavioral performance than I/R mice (P = 0.0171, 0.0241, 0.0219 in 1, 2 and 3 days after reperfusion, respectively). Additionally, supplementation with TDZD reduced the brain infarct volume compared with that of the I/R mice [28.9 (1.9%) vs. 43.1 (3.1%), P = 0.0053]. No statistically significant difference was detected between the I/R and I/R + vehicle groups.

Neuronal cell apoptosis in the ischemic penumbra is shown in Fig. 3D. At 72 h after reperfusion, the number of TUNEL-positive cells in the SPC group was significantly decreased compared with that of the I/R group (P = 0.0186). Moreover, the number of TUNEL-positive cells in the TDZD-treated group was reduced compared to that in the I/R + vehicle group (P = 0.0314).

GSK-3β Inhibition Promoted M2 Microglia/Macrophage Polarization in the Ischemic Hemisphere at 7 Days After Reperfusion

As shown in Supplementary Fig. S2, the GSK-3β phosphorylation was measured by Western blots. The phosphorylation of GSK-3β at Ser9 was decreased in the vehicle-treated I/R groups compared with the control group (P = 0.0210), but this reduction was ameliorated by TDZD supplementation (TDZD + I/R vs. I/R + vehicle, P = 0.0045).

As illustrated in Fig. 4, the protein (Fig. 4A) and mRNA (Fig. 4B) levels of TNF-α, IL-1β and iNOS were examined by ELISA kits. The protein levels of these proinflammatory factors were exaggerated in the I/R group compared with the control group but was reduced in the SPC group (P = 0.0279, 0.0054, 0.0170, respectively). The administration of TDZD also reduced the expression of these M1 markers compared with those of the I/R group (P = 0.0078, 0.0030, 0.0049, respectively). No significant difference was detected between the TDZD + I/R and Sevo + vehicle groups. As shown in Fig. 4B, the mRNA expression of

the M1 proinflammatory factors was significantly elevated in the ischemic penumbra but was reduced when mice received SPC. The TDZD treatment also reduced the elevated mRNA expression of these proinflammatory factors (TDZD + I/R vs. I/R: P = 0.0206, < 0.001, 0.0157, respectively). As shown in Fig. 4C, the mRNA levels of the M2 gene markers in the Sevo + I/R group were higher than those in the I/R group. TDZD also increased the level of these anti-inflammatory factors (CD206, YM1/2, arginase-1: P < 0.001).

SPC and TDZD Treatment Increased Nuclear Translocation of Nrf2, And Nrf2 Deficiency Reversed the Neuroprotective Effect Produced by TDZD Supplementation

As shown in Fig. 5A, the Nrf2 protein content in the nucleus was increased in the SPC and TDZD-treated groups compared to the I/R group (P = 0.0222 and 0.0323, respectively). As shown in Fig. 5A (right panel), double immunofluorescence staining showed that the Nrf2 protein was increased by SPC and TDZD treatment, and Nrf2-positive cells were mainly neurons.

To further determine the relationship between GSK-3β and Nrf2 under SPC, we applied an Nrf2-shRNA (AAV-Nrf2 and its control AAV-GFP) in this experiment to silence the expression of Nrf2. As shown in Fig. 5B, TDZD significantly ameliorated the neurobehavioral outcome, while Nrf2 knockdown reversed this improvement, as judged by the neurological scores at 1 day, 3 days and 7 days in the TDZD + AAV-Nrf2 group compared with the TDZD group at parallel timepoints (P = 0.0193, 0.0219, and 0.0132, respectively, for each comparison). Consistent with the change in neurological outcome, the infarct size in the TDZD + AAV-Nrf2 group was larger than that in the TDZD group at 7 days after reperfusion [figure 5C, 32.51 (1.51%) vs. 24.34 (1.2%), P = 0.0049]. However, no significant difference was detected between the I/R and I/R + vehicle groups. At 72 h after reperfusion, the reduced number of TUNEL-positive cells in the TDZD group was significantly reversed by the introduction of the Nrf2 mutation (Fig. 5D, P = 0.0265).

Knockdown of Nrf2 Abolished the Promoted M2 Microglia/Macrophage Polarization Produced by GSK-3β Inhibition

AAV-Nrf2 microinjection led to a significant reduction of Nrf2 positive cells (see Supplementary Fig. S3). The protein levels of these proinflammatory factors were reduced in the TDZD group compared with the I/R group, while Nrf2 knockdown prevented this reduction (Fig. 6A; P = 0.0241, 0.0183, 0.0081). As shown in Fig. 6B, the mRNA expression of the M1 proinflammatory factors was significantly reduced in the ischemic penumbra treated with TDZD; however, this attenuated expression was abolished by the administration of AAV-Nrf2 (P = 0.0073, 0.0407, 0.0205, respectively). As shown in Fig. 6C, the mRNA levels of anti-inflammatory factors were higher in the TDZD group than in the I/R group, while Nrf2 deficiency prevented this improvement (CD206, YM1/2, and arginase-1: P < 0.001, = 0.0150, and 0.0127, respectively).

Knockdown of Nrf2 Reversed M2 Microglia/Macrophage Polarization and Abolished The Inhibition of Reactive Oxygen Species Generation Produced by TDZD Treatment

Representative images of flow cytometry that were used to examine the ratio of M1- and M2-positive microglia are shown in Fig. 7A. As shown in Fig. 7B, activated M1 microglia (CD86+) intensely accumulated in the ischemic penumbra compared with the ipsilateral hemisphere of the control group ($P < 0.001$), while SPC and TDZD treatment significantly reduced this accumulation ($P < 0.001$ and $P = 0.0003$, respectively). However, this reduction was reversed by mutation of Nrf2 ($P = 0.003$). As shown in Fig. 7C, the percentage of M2-positive microglia (CD206+) was not affected by I/R surgery, but both SPC and TDZD treatment increased this ratio compared with that of the I/R group (Sevo + I/R: $P < 0.001$, TDZD + I/R: $P = 0.0013$). As expected, supplementation with AAV-Nrf2 reversed the improved accumulation of M2 microglia ($P = 0.016$). No significant difference was detected between the TDZD and TDZD + AAV-GFP groups in the percentage of M1 or M2 microglia.

As indicated in Fig. 7D, the generation of reactive oxygen species (ROS) was measured by DHE staining. Increased levels of DHE-positive cells were detected in the I/R group, while both SPC and TDZD treatment reduced this increase (Fig. 7E, Sevo + I/R: $P = 0.0257$, TDZD + I/R: $P = 0.0323$). Moreover, mutation of Nrf2 reversed the reduced DHE-positive cells induced by TDZD ($P = 0.0131$).

Discussion

Using an in vivo transient focal cerebral I/R model and two in vitro OGD/LPS-induced injury models, the current study demonstrated that GSK-3β phosphorylation-regulated Nrf2 activation was involved in the improvement of M2 microglia polarization induced by SPC after brain ischemia. SPC reduced the level of M1 marker genes and promoted M2 marker mRNA expression. SPC also increased the phosphorylation of GSK-3β, and supplementation with the GSK-3β inhibitor TDZD mimicked the effect of SPC on microglia polarization and ischemic tolerance. Moreover, preconditioning with sevoflurane or administration of TDZD increased Nrf2 nuclear translocation, reduced the infarct volume, improved neurological function, attenuated cellular apoptosis, reduced the mRNA levels of proinflammatory factors, decreased super-oxidants generation and promoted the mRNA expression of anti-inflammatory factors after brain ischemia. However, these benefits induced by SPC or TDZD were reversed by the knockdown of Nrf2. Taken together, the results of this study identified a novel mechanism of SPC-induced neuroprotection against cerebral I/R injury.

Immune cells within the brain, including microglia, appear to be heterogeneous with diverse functional phenotypes that range from immuno-enhanced M1 phenotypes to anti-inflammatory M2 phenotypes[35, 36]. The activation of the immunosuppressive M2 phenotype and the attenuation of the proinflammatory M1 phenotype (M1/M2 switching) play a vital role in the stimulation of neuroprotective mechanisms mediating the inhibition of neuroinflammation to protect neurons from injury[12, 16, 36, 37]. Some preconditioning therapeutics, such as sevoflurane, elicit neuroprotective effects by attenuating inflammatory process-related signals, including TNF-α and Nuclear Factor κB[29, 38–40]. However, in the brain, the mechanism underlying the regulatory effect of SPC on the M1/M2 shift remains unclear. Considerable evidence has shown that the shift of M1/M2 could be indicated by changes in the expression of some inflammatory factors, whereas TNF-α, IL-1β and iNOS are related to the M1

phenotype, while CD-206, YM1/2 and arginase-1 are in correlation with the M2 phenotype[10, 35, 41–44]. Therefore, in the current study, we first measured the mRNA expression of M1 and M2 gene markers. SPC induced a significant increase in CD-206, YM1/2 and arginase-1 but an obvious reduction of TNF-α, IL-1β and iNOS mRNA. These findings are consistent with previous studies related to the M1/M2 paradigm of microglial activation in several neurodegenerative diseases, including cerebral ischemic stroke. To further clarify the protection of SPC against M1/M2-shift regulated immunopathogenesis, we employed an OGD-induced injury model and LPS-stimulation model in primary cortical microglia culture and found that SPC also prevented M1 marker mRNA expression and enhanced the expression of M2 genes in these two in vitro models, which was consistent with other studies using different protective stimuli[12, 36].

Another major finding of this study is that SPC engages M2 microglia activation in a GSK-3β phosphorylation-dependent manner. In addition to regulating physiological processes such as glucose metabolism, cellular development and differentiation, GSK-3β is also a target that prevents ischemic insult. The phosphorylation of GSK-3β at Ser9, which indicates an inactive status, enables cells to resist various pathophysiological injuries[45]. In the CNS, GSK-3β was reported to affect the microglial activation by modulating a cascade of signals[46–49]. Thus, we employed a GSK-3β inhibitor at Ser9, TDZD, and found that both SPC and TDZD elicited a neuroprotective effect by improving M2 marker gene expression and reducing M1 marker mRNA expression[50, 51]. These results indicated that the SPC-mediated improvement of M2 phenotype polarization was GSK-3β inactivation dependent. Our results were also consistent with those of previous studies showing that GSK-3β participated in the cerebral ischemic tolerance produced by preconditioning agents and that GSK-3β phosphorylation was crucial to maintaining M2 microglial activation.

SPC protects the brain by GSK-3β inhibitor-enhanced M2 microglial activation, but how GSK-3β regulates M2 phenotype polarization is still being debated. Previous studies in our group and others found that the Nrf2-regulated antioxidant response element (ARE) participated in the neuroprotective and antioxidant effects of SPC, and this study also revealed that SPC-induced Nrf2 activation may be partly mediated by other Keap1-independent mechanisms[3, 52]. GSK-3β has been reported to function as a supplement to the Keap1-Nrf2 degradation mechanism and to influence the nuclear exclusion and inactivation of Nrf2[17, 22]. GSK-3β was shown to downregulate the Nrf2/ARE pathway and may represent a new treatment target against cerebral I/R injury[15, 17, 25]. In this study, we found that GSK-3β inhibition, which was induced by TDZD or SPC, increased Nrf2 translocation, while knockdown of Nrf2 by AAV-shRNA administration reversed the enhanced M2 phenotype polarization and neuroprotective effect of TDZD. This finding provides a plausible explanation for how GSK-3β could regulate M2 microglia activation after SPC. Additionally, considering the relationship between oxidative stress and inflammatory pathways and that these signalling cascades could be regulated by Nrf2, we further investigated whether the knockdown of Nrf2 reversed the attenuated M1-labelled cells and the enhanced M2-labelled cells induced by SPC or TDZD. These results were consistent with the results of the analysis of super-oxidants.

Conclusions

In summary, this study demonstrated that GSK-3β phosphorylation-mediated Nrf2 activation is involved in SPC-induced cerebral ischemic tolerance by improving M2 microglia polarization after cerebral I/R. These investigations may reveal a novel mechanism of SPC-induced neuroprotection.

Abbreviations

AAV
adeno-associated virus
CNS
central nervous system
DHE
dihydroethidium
GSK-3β
glycogen synthase kinase-3β
IL-1β
interleukin-1β
iNOS
inducible nitric oxide synthase
MCAO
middle cerebral artery occlusion
LDH
lactate dehydrogenase
LPS
lipopolysaccharide
I/R
ischemia/reperfusion
MTT
3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazol ium bromide
Keap1
kelch-like ECH-associated protein 1
OGD
oxygen-glucose deprivation
rCBF
regional cerebral blood flow
SPC
sevoflurane preconditioning
TDZD
4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione
TNF-α
tumour necrosis factor-α

TUNEL
terminal deoxynucleotidyl transferase deoxyuridine triphosphate-biotin nick-end labelling

Declarations

Acknowledgment

We thank American Journal Experts (AJE) for assisting in the preparation of this manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 81700145 for MC, No. 81603408 to SSS, No. 81571351 to HLD, and 81771411 for WGH) and the Fund for International Cooperation and Exchange of the National Natural Science Foundation of China (81620108012 to HLD).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

MC and WGH designed and conducted the experiments, collected and analysed the data and wrote the paper. SSS, SQW, LT and BBD participated in the experiments, data collection and analysis. MC, HLD and WGH supervised the study and helped with the study design and data analysis and completion of the manuscript. All authors approved the final manuscript.

Ethics approval

All animal experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

None.

Competing interests

The authors declare that they have no competing interests.

References

1. Wechsler LR: **Intravenous thrombolytic therapy for acute ischemic stroke.** *N Engl J Med* 2011, **364:**2138-2146.
2. Tong L, Cai M, Huang Y, Zhang H, Su B, Li Z, Dong H: **Activation of K(2)P channel-TREK1 mediates the neuroprotection induced by sevoflurane preconditioning.** *Br J Anaesth* 2014, **113:**157-167.
3. Cai M, Tong L, Dong B, Hou W, Shi L, Dong H: **Kelch-like ECH-associated Protein 1-dependent Nuclear Factor-E2-related Factor 2 Activation in Relation to Antioxidation Induced by Sevoflurane Preconditioning.** *Anesthesiology* 2017, **126:**507-521.
4. Adamczyk S, Robin E, Simerabet M, Kipnis E, Tavernier B, Vallet B, Bordet R, Lebuffe G: **Sevoflurane pre- and post-conditioning protect the brain via the mitochondrial K ATP channel.** *Br J Anaesth* 2010, **104:**191-200.
5. Chen Y, Nie H, Tian L, Tong L, Deng J, Zhang Y, Dong H, Xiong L: **Sevoflurane preconditioning-induced neuroprotection is associated with Akt activation via carboxy-terminal modulator protein inhibition.** *Br J Anaesth* 2015, **114:**327-335.
6. Jayaraj RL, Azimullah S, Beiram R, Jalal FY, Rosenberg GA: **Neuroinflammation: friend and foe for ischemic stroke.** *J Neuroinflammation* 2019, **16:**142.
7. Glezer I, Simard AR, Rivest S: **Neuroprotective role of the innate immune system by microglia.** *Neuroscience* 2007, **147:**867-883.
8. Block ML, Zecca L, Hong JS: **Microglia-mediated neurotoxicity: uncovering the molecular mechanisms.** *Nat Rev Neurosci* 2007, **8:**57-69.
9. Xiong XY, Liu L, Yang QW: **Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke.** *Prog Neurobiol* 2016, **142:**23-44.
10. Liu X, Liu J, Zhao S, Zhang H, Cai W, Cai M, Ji X, Leak RK, Gao Y, Chen J, Hu X: **Interleukin-4 Is Essential for Microglia/Macrophage M2 Polarization and Long-Term Recovery After Cerebral Ischemia.** *Stroke* 2016, **47:**498-504.
11. Tang Y, Le W: **Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases.** *Mol Neurobiol* 2016, **53:**1181-1194.
12. Wang Y, Huang Y, Xu Y, Ruan W, Wang H, Zhang Y, Saavedra JM, Zhang L, Huang Z, Pang T: **A Dual AMPK/Nrf2 Activator Reduces Brain Inflammation After Stroke by Enhancing Microglia M2 Polarization.** *Antioxid Redox Signal* 2018, **28:**141-163.
13. Valerio A, Bertolotti P, Delbarba A, Perego C, Dossena M, Ragni M, Spano P, Carruba MO, De Simoni MG, Nisoli E: **Glycogen synthase kinase-3 inhibition reduces ischemic cerebral damage, restores impaired mitochondrial biogenesis and prevents ROS production.** *J Neurochem* 2011, **116:**1148-1159.
14. Wang Y, Ge C, Chen J, Tang K, Liu J: **GSK-3beta inhibition confers cardioprotection associated with the restoration of mitochondrial function and suppression of endoplasmic reticulum stress in sevoflurane preconditioned rats following ischemia/reperfusion injury.** *Perfusion* 2018, **33:**679-686.
15. Wang M, Wang K, Gao X, Zhao K, Chen H, Xu M: **Anti-inflammatory effects of isoalantolactone on LPS-stimulated BV2 microglia cells through activating GSK-3beta-Nrf2 signaling pathway.** *Int*

Immunopharmacol 2018, **65:**323-327.

16. Wang G, Shi Y, Jiang X, Leak RK, Hu X, Wu Y, Pu H, Li WW, Tang B, Wang Y, et al: **HDAC inhibition prevents white matter injury by modulating microglia/macrophage polarization through the GSK3beta/PTEN/Akt axis.** *Proc Natl Acad Sci U S A* 2015, **112:**2853-2858.
17. Dai X, Yan X, Zeng J, Chen J, Wang Y, Chen J, Li Y, Barati MT, Wintergerst KA, Pan K, et al: **Elevating CXCR7 Improves Angiogenic Function of EPCs via Akt/GSK-3beta/Fyn-Mediated Nrf2 Activation in Diabetic Limb Ischemia.** *Circ Res* 2017, **120:**e7-e23.
18. Cai L, Li Y, Zhang Q, Sun H, Yan X, Hua T, Zhu Q, Xu H, Fu H: **Salidroside protects rat liver against ischemia/reperfusion injury by regulating the GSK-3beta/Nrf2-dependent antioxidant response and mitochondrial permeability transition.** *Eur J Pharmacol* 2017, **806:**32-42.
19. Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A, Hayes JD: **Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity.** *Oncogene* 2013, **32:**3765-3781.
20. Cuadrado A: **Structural and functional characterization of Nrf2 degradation by glycogen synthase kinase 3/beta-TrCP.** *Free Radic Biol Med* 2015, **88:**147-157.
21. Cuadrado A, Kugler S, Lastres-Becker I: **Pharmacological targeting of GSK-3 and NRF2 provides neuroprotection in a preclinical model of tauopathy.** *Redox Biol* 2018, **14:**522-534.
22. Rada P, Rojo AI, Evrard-Todeschi N, Innamorato NG, Cotte A, Jaworski T, Tobon-Velasco JC, Devijver H, Garcia-Mayoral MF, Van Leuven F, et al: **Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis.** *Mol Cell Biol* 2012, **32:**3486-3499.
23. Kensler 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.
24. Zhang M, An C, Gao Y, Leak RK, Chen J, Zhang F: **Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection.** *Prog Neurobiol* 2013, **100:**30-47.
25. Chen X, Liu Y, Zhu J, Lei S, Dong Y, Li L, Jiang B, Tan L, Wu J, Yu S, Zhao Y: **GSK-3beta downregulates Nrf2 in cultured cortical neurons and in a rat model of cerebral ischemia-reperfusion.** *Sci Rep* 2016, **6:**20196.
26. Wei H, Yao X, Yang L, Wang S, Guo F, Zhou H, Marsicano G, Wang Q, Xiong L: **Glycogen synthase kinase-3beta is involved in electroacupuncture pretreatment via the cannabinoid CB1 receptor in ischemic stroke.** *Mol Neurobiol* 2014, **49:**326-336.
27. Su F, Guo AC, Li WW, Zhao YL, Qu ZY, Wang YJ, Wang Q, Zhu YL: **Low-Dose Ethanol Preconditioning Protects Against Oxygen-Glucose Deprivation/Reoxygenation-Induced Neuronal Injury By Activating Large Conductance, Ca(2+)-Activated K(+) Channels In Vitro.** *Neurosci Bull* 2017, **33:**28-40.
28. Velly LJ, Canas PT, Guillet BA, Labrande CN, Masmejean FM, Nieoullon AL, Gouin FM, Bruder NJ, Pisano PS: **Early anesthetic preconditioning in mixed cortical neuronal-glial cell cultures subjected to oxygen-glucose deprivation: the role of adenosine triphosphate dependent potassium channels and reactive oxygen species in sevoflurane-induced neuroprotection.** *Anesth Analg* 2009, **108:**955-963.

29. Suter D, Spahn DR, Blumenthal S, Reyes L, Booy C, Z'Graggen B R, Beck-Schimmer B: **The immunomodulatory effect of sevoflurane in endotoxin-injured alveolar epithelial cells.** *Anesth Analg* 2007, **104:**638-645.
30. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L: **Cell Viability Assays.** In *Assay Guidance Manual.* Edited by Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin C, Baell J, Bejcek B, Caaveiro JMM, Chung TDY*, et al*. Bethesda (MD); 2004
31. Garcia JH, Wagner S, Liu KF, Hu XJ: **Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation.** *Stroke* 1995, **26:**627-634; discussion 635.
32. Swanson RA, Sharp FR: **Infarct measurement methodology.** *J Cereb Blood Flow Metab* 1994, **14:**697-698.
33. Ashwal S, Tone B, Tian HR, Cole DJ, Pearce WJ: **Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion.** *Stroke* 1998, **29:**1037-1046; discussion 1047.
34. Dugan LL, You YH, Ali SS, Diamond-Stanic M, Miyamoto S, DeCleves AE, Andreyev A, Quach T, Ly S, Shekhtman G, et al: **AMPK dysregulation promotes diabetes-related reduction of superoxide and mitochondrial function.** *J Clin Invest* 2013, **123:**4888-4899.
35. Espinosa-Garcia C, Sayeed I, Yousuf S, Atif F, Sergeeva EG, Neigh GN, Stein DG: **Stress primes microglial polarization after global ischemia: Therapeutic potential of progesterone.** *Brain Behav Immun* 2017, **66:**177-192.
36. Xu Y, Xu Y, Wang Y, Wang Y, He L, Jiang Z, Huang Z, Liao H, Li J, Saavedra JM, et al: **Telmisartan prevention of LPS-induced microglia activation involves M2 microglia polarization via CaMKKbeta-dependent AMPK activation.** *Brain Behav Immun* 2015, **50:**298-313.
37. Yao X, Liu S, Ding W, Yue P, Jiang Q, Zhao M, Hu F, Zhang H: **TLR4 signal ablation attenuated neurological deficits by regulating microglial M1/M2 phenotype after traumatic brain injury in mice.** *J Neuroimmunol* 2017, **310:**38-45.
38. Wang H, Lu S, Yu Q, Liang W, Gao H, Li P, Gan Y, Chen J, Gao Y: **Sevoflurane preconditioning confers neuroprotection via anti-inflammatory effects.** *Front Biosci (Elite Ed)* 2011, **3:**604-615.
39. Watanabe K, Iwahara C, Nakayama H, Iwabuchi K, Matsukawa T, Yokoyama K, Yamaguchi K, Kamiyama Y, Inada E: **Sevoflurane suppresses tumour necrosis factor-alpha-induced inflammatory responses in small airway epithelial cells after anoxia/reoxygenation.** *Br J Anaesth* 2013, **110:**637-645.
40. Dang DD, Saiyin H, Yu Q, Liang WM: **Effects of sevoflurane preconditioning on microglia/macrophage dynamics and phagocytosis profile against cerebral ischemia in rats.** *CNS Neurosci Ther* 2018, **24:**564-571.
41. Tu HJ, Kang KH, Ho SY, Liou HC, Liou HH, Lin CP, Fu WM: **Leukemia inhibitory factor (LIF) potentiates antinociception activity and inhibits tolerance induction of opioids.** *Br J Anaesth* 2016, **117:**512-520.
42. Madsen DH, Leonard D, Masedunskas A, Moyer A, Jurgensen HJ, Peters DE, Amornphimoltham P, Selvaraj A, Yamada SS, Brenner DA, et al: **M2-like macrophages are responsible for collagen**

degradation through a mannose receptor-mediated pathway. *J Cell Biol* 2013, **202:**951-966.

43. Chang NC, Hung SI, Hwa KY, Kato I, Chen JE, Liu CH, Chang AC: **A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin.** *J Biol Chem* 2001, **276:**17497-17506.
44. Bronte V, Zanovello P: **Regulation of immune responses by L-arginine metabolism.** *Nat Rev Immunol* 2005, **5:**641-654.
45. Zhou H, Wang H, Ni M, Yue S, Xia Y, Busuttil RW, Kupiec-Weglinski JW, Lu L, Wang X, Zhai Y: **Glycogen synthase kinase 3beta promotes liver innate immune activation by restraining AMP-activated protein kinase activation.** *J Hepatol* 2018, **69:**99-109.
46. Khan MS, Ali T, Kim MW, Jo MH, Chung JI, Kim MO: **Anthocyanins Improve Hippocampus-Dependent Memory Function and Prevent Neurodegeneration via JNK/Akt/GSK3beta Signaling in LPS-Treated Adult Mice.** *Mol Neurobiol* 2019, **56:**671-687.
47. Xu H, Li J, Wang Z, Feng M, Shen Y, Cao S, Li T, Peng Y, Fan L, Chen J, et al: **Methylene blue attenuates neuroinflammation after subarachnoid hemorrhage in rats through the Akt/GSK-3beta/MEF2D signaling pathway.** *Brain Behav Immun* 2017, **65:**125-139.
48. Zhou K, Zhong Q, Wang YC, Xiong XY, Meng ZY, Zhao T, Zhu WY, Liao MF, Wu LR, Yang YR, et al: **Regulatory T cells ameliorate intracerebral hemorrhage-induced inflammatory injury by modulating microglia/macrophage polarization through the IL-10/GSK3beta/PTEN axis.** *J Cereb Blood Flow Metab* 2017, **37:**967-979.
49. Xia Y, Rao J, Yao A, Zhang F, Li G, Wang X, Lu L: **Lithium exacerbates hepatic ischemia/reperfusion injury by inhibiting GSK-3beta/NF-kappaB-mediated protective signaling in mice.** *Eur J Pharmacol* 2012, **697:**117-125.
50. Martinez A, Alonso M, Castro A, Perez C, Moreno FJ: **First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease.** *J Med Chem* 2002, **45:**1292-1299.
51. Lipina TV, Wang M, Liu F, Roder JC: **Synergistic interactions between PDE4B and GSK-3: DISC1 mutant mice.** *Neuropharmacology* 2012, **62:**1252-1262.
52. Lee H, Park YH, Jeon YT, Hwang JW, Lim YJ, Kim E, Park SY, Park HP: **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.** *Br J Anaesth* 2015, **114:**307-318.

Table 1

Gene Name	Sense (5’-3’)	Anti-sense (5’-3’)
GAPDH	AACTTTGGCATTGTG GAAGG	GGATGCAGGGATGATGTTCT
TNF-α	GCTGAGCTCAAACCCTGGTA	CGGACTCCGCAAAGTCTAAG
IL-1β	TGTGAAATGCCATTTGA	GGTCAAAGGTTTGGAAGCAG
iNOS	CCCAGAGTTCCAGCTTCTGG	CCAAGCCCCTCACCATTATCT
CD-206	CTTCGGGCCTTTGGAATAAT	TAGAAGAGCCCTTGGGTTGA
YM1/2	CAGGGTAATGAGTGGGTTGG	CACGGCACCTCCTAAATTGT
Arg1	CTGGTCGGTTTGATGCTA	TGCTTAGCTCTGTCTGCTTTGC

Table 1. The primers of anti-inflammation and pro-inflammation factors used in this study.

Figures

Figure 1

SPC reduced the mRNA expression of proinflammatory factors and increased the mRNA levels of anti-inflammatory factors after OGD or LPS stimulation. (A) Effect of SPC on cell viability and plasma lactate dehydrogenase (LDH) release levels in primary cortical microglia after OGD. (B) The mRNA expression of proinflammatory factors (TNF-α, IL-1β and iNOS) following OGD. (C) The mRNA levels of anti-inflammatory factors (CD-206, YM1/2, arginase-1) following OGD. (D) The measurement of cell viability and LDH release after LPS stimulation. (E) The mRNA expression of proinflammatory factors (TNF-α, IL-1β and iNOS) following LPS. (F) The mRNA levels of anti-inflammatory factors (CD-206, YM1/2, arginase-1) after LPS. n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. OGD/LPS. One way ANOVA with Tukey’s post hoc test was used for statistic analysis. OGD = oxygen-glucose deprivation; LPS = lipopolysaccharide; Sevo = sevoflurane preconditioning; TNF-α = Tumour necrosis factor-α; IL-1β = Interleukin-1β; iNOS = inducible nitric oxide synthase.

Figure 2

. SPC reduced the mRNA expression of proinflammatory factors and increased the mRNA levels of anti-inflammatory factors at 7 and 14 days after reperfusion. (A) The mRNA expression of M1 marker genes (TNF-α, IL-1β and iNOS) in the penumbra 7 days following reperfusion. (B) The mRNA levels of M2 marker genes (CD-206, YM1/2, arginase-1) in the penumbra 7 days following reperfusion. (C) The mRNA

levels of M1 marker genes (TNF-α, IL-1β and iNOS) in the penumbra 14 days after reperfusion. (D) The mRNA expression of M2 marker genes (CD-206, YM1/2, arginase-1) in the penumbra 14 days after reperfusion. n = 6,* P < 0.05, ** P < 0.01, *** P < 0.001 vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. Sevo + I/R. One way ANOVA with Tukey's post hoc test was used for statistic analysis. Sevo = sevoflurane preconditioning; I/R = ischemia/reperfusion; TNF-α = Tumour necrosis factor-α; IL-1β = Interleukin-1β; iNOS = inducible nitric oxide synthase.

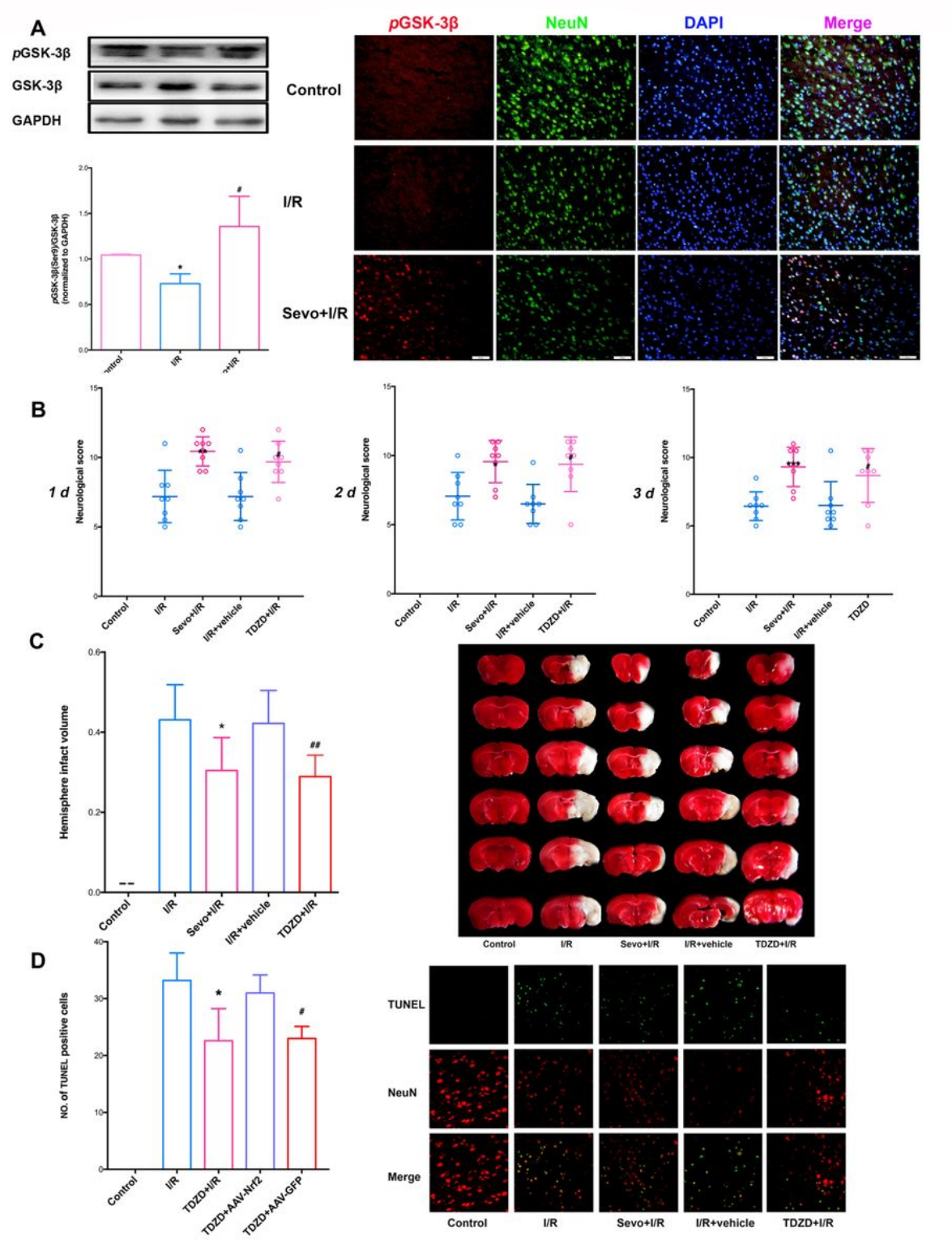

Figure 3

SPC increased the phosphorylation of GSK-3β, and supplementation with TDZD mimicked the neuroprotection induced by SPC. (A) Western blot analysis for GSK-3β phosphorylation at Ser9. (n = 6). Immunofluorescence staining of phosphorylated GSK-3β (pGSK-3β) and neuronal nuclei (NeuN). SPC increased the number of pGSK-3β-positive neurons compared with I/R (right panel). Scale bar = 10 μm. (B) The TDZD-mediated improvement in neurological manifestations at 1, 3 and 7 days after reperfusion. Each symbol presents the score of a single rat. (C) Comparisons of the percentages of infarction size among the control, I/R, Sevo + I/R, I/R + vehicle and TDZD + I/R groups (n = 8). (D) Cellular apoptosis was measured by TUNEL staining in the ischemic penumbra. The panel below is the quantitative statistical result of TUNEL-positive cells in each group (n = 5). Scale bar = 20 μm.* $P < 0.05$, ** $P < 0.01$ vs. the I/R group; # $P < 0.05$, ## $P < 0.01$ vs. the I/R + vehicle group. One way ANOVA with Tukey's post hoc test was used for statistic analysis. Sevo = sevoflurane preconditioning; I/R = ischemia/reperfusion; TDZD = 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; TUNEL = terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling.

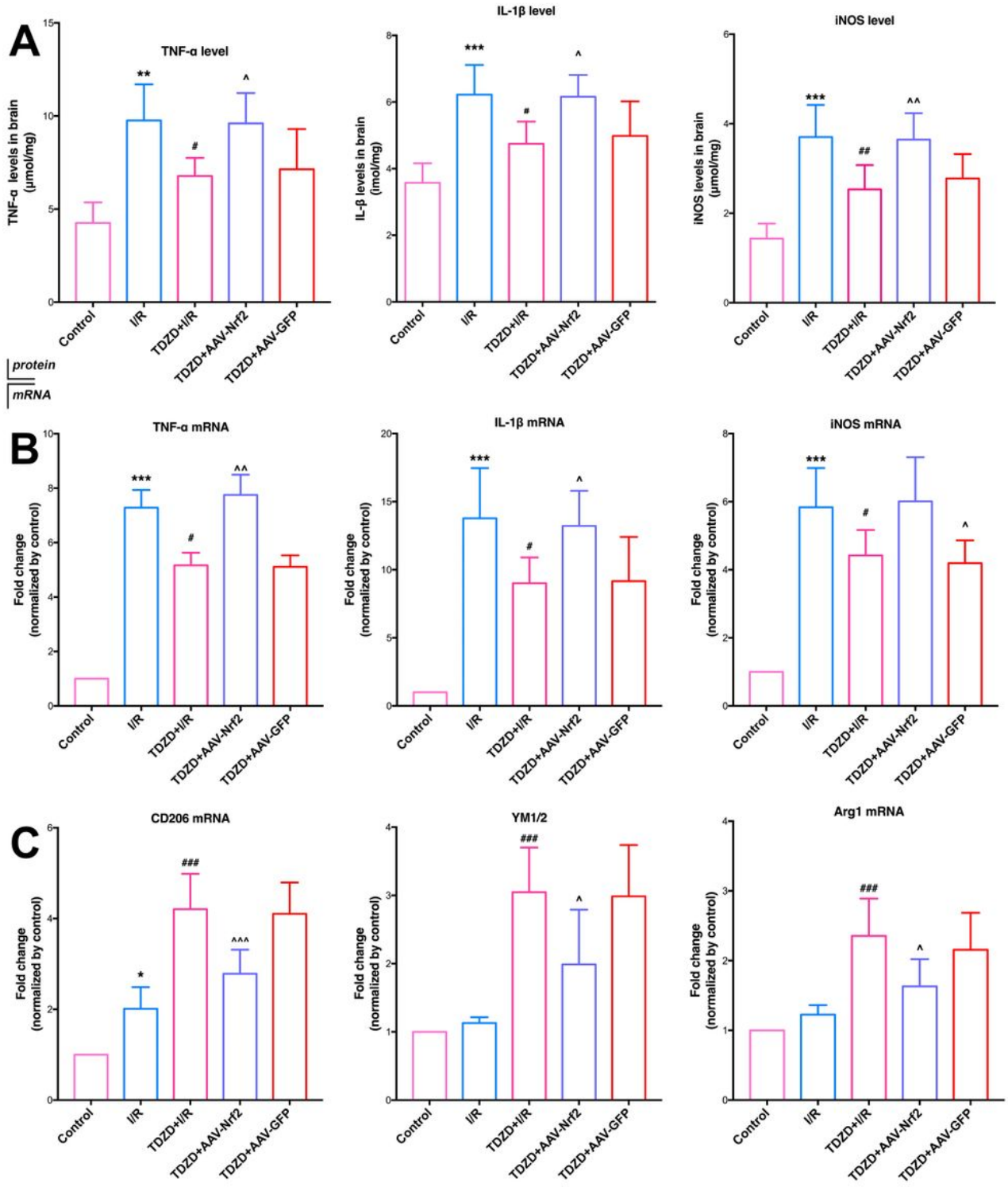

Figure 4

TDZD reduced the mRNA expression of proinflammatory factors and increased the mRNA level of anti-inflammatory factors after ischemia/reperfusion. (A) Effect of TDZD on the protein expression of proinflammatory factors (TNF-α, IL-1β and iNOS). (B) The mRNA expression of proinflammatory factors (TNF-α, IL-1β and iNOS) following 7 days of reperfusion. (C) The mRNA levels of anti-inflammatory factors (CD-206, YM1/2, arginase-1) 7 days after reperfusion. n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001

vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the I/R group. One way ANOVA with Tukey's post hoc test was used for statistic analysis. Sevo = sevoflurane preconditioning; I/R = ischemia/reperfusion; TNF-α = Tumour necrosis factor-α; IL-1β= Interleukin-1β; iNOS = inducible nitric oxide synthase.

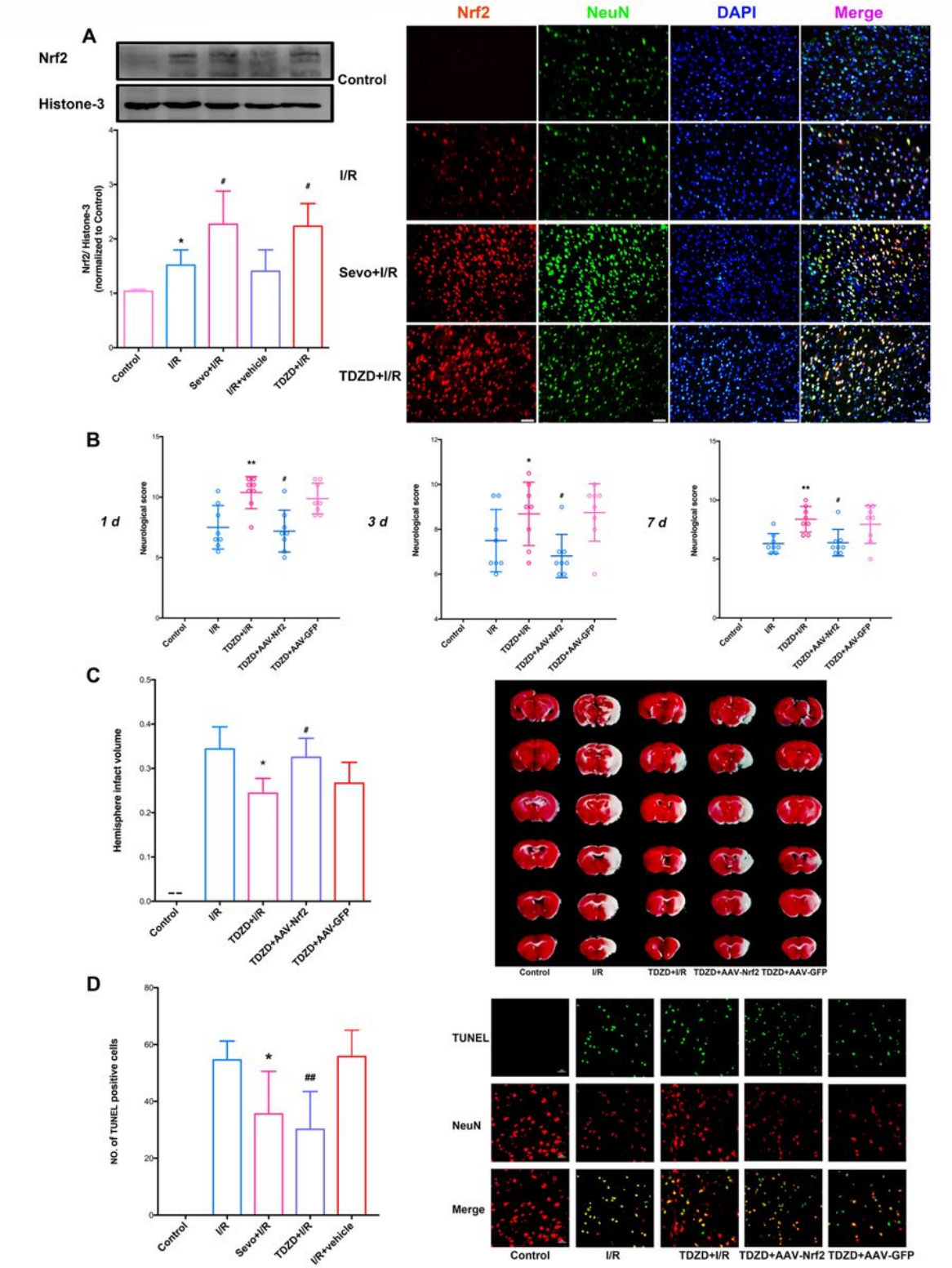

Figure 5

SPC and TDZD treatment increased Nrf2 nuclear translocation; knockdown of Nrf2 reversed the neuroprotection induced by TDZD. (A) Western blot analysis of Nrf2 translocation. (n = 6 per group). Immunofluorescence staining of Nrf2 expression and neuronal nuclei (NeuN). SPC and TDZD increased the number of Nrf2-positive neurons compared with I/R (right panel). Scale bar = 10 μm. (B) Nrf2 mutation prevented the ameliorated neurological manifestations induced at 1, 3 and 7 days after reperfusion. Each symbol presents the score of a single rat. (C) Comparisons of the percentages of infarction size among the control, I/R, TDZD + I/R, TDZD + AAV-Nrf2 and TDZD + AAV-GFP groups (n = 8). (D) Cellular apoptosis was measured by TUNEL staining in the ischemic penumbra. The panel below is the quantitative statistical results of TUNEL-positive cells in each group (n = 5). Scale bar = 20 μm. * $P < 0.05$, ** $P < 0.01$ vs. the I/R group; # $P < 0.05$ vs. the TDZD + I/R group. One way ANOVA with Tukey's post hoc test was used for statistic analysis. I/R = ischemia/reperfusion; TDZD = 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; AAV-Nrf2 = adeno-associated virus induced Nrf2-shRNA; TUNEL = terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling.

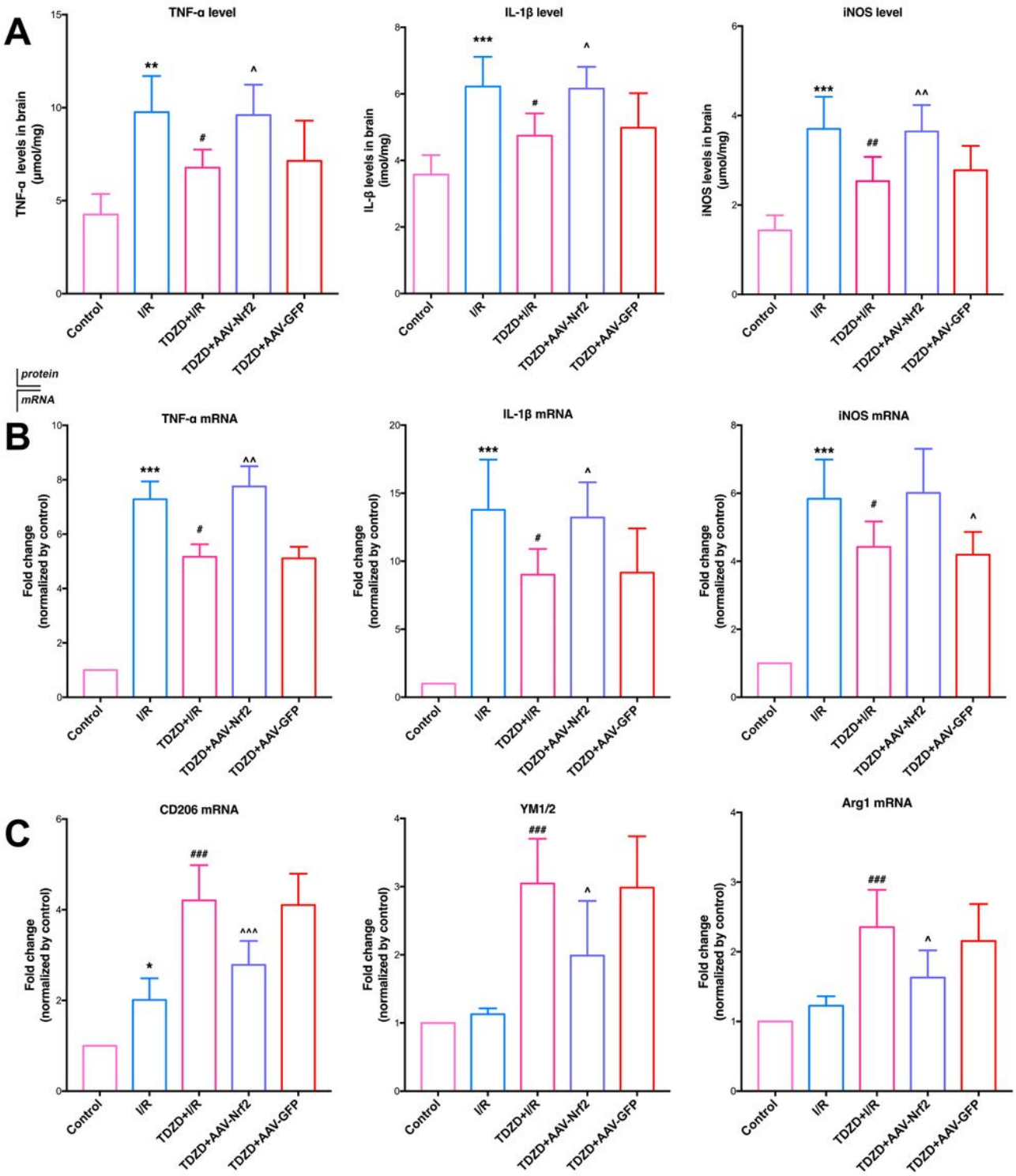

Figure 6

The deletion of Nrf2 reversed the reduced mRNA expression of proinflammatory factors and increased the mRNA level of anti-inflammatory factors induced by TDZD administration after ischemia/reperfusion. (A) Effect of AAV-Nrf2 on the protein expression of proinflammatory factors (TNF-α, IL-1β and iNOS). (B) The mRNA expression of proinflammatory factors (TNF-α, IL-1β and iNOS) following 7 days of reperfusion. (C) The mRNA levels of anti-inflammatory factors (CD-206, YM1/2, arginase-1) 7 days after

reperfusion. n = 6, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the I/R group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the I/R group; ^ P < 0.05, ^^ P < 0.01, ^^^ P < 0.001. One way ANOVA with Tukey's post hoc test was used for statistic analysis. I/R = ischemia/reperfusion; TDZD = 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; AAV-Nrf2 = adeno-associated virus induced Nrf2-shRNA; TNF-α = Tumour necrosis factor-α; IL-1β= Interleukin-1β; iNOS = inducible nitric oxide synthase.

Figure 7

SPC and TDZD treatment alleviated M1 microglial but increased M2 microglial polarization and reduced the generation of ROS. These effects were reversed by the deficiency of Nrf2. (A) Flow cytometry analysis of CD86+ (M1 microglia) and CD206+ (M2 microglia) cells in the ischemic penumbra 7 days after reperfusion. (B, C) The quantitative statistical results of CD206-positive cells (B) and CD86- (C) in each group (n = 3). (D) Representative immunofluorescence micrographs showing DHE staining for ROS in each group. Scale bar = 15 μm. (E) The quantitative analysis of DHE-positive cells. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the I/R group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the I/R group; ^ P < 0.05, ^^ P < 0.01, ^^^ P < 0.001. One way ANOVA with Tukey's post hoc test was used for statistic analysis. I/R = ischemia/reperfusion; Sevo = sevoflurane preconditioning; TDZD = 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; AAV-Nrf2 = adeno-associated virus induced Nrf2-shRNA; DHE = dihydroethidium.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementmeterials.docx