Inhibition of N-myc Downstream–regulated Gene-2 Is Involved in an Astrocyte-specific Neuroprotection Induced by Sevoflurane Preconditioning

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ABSTRACT

Background: Mechanism of sevoflurane preconditioning–induced cerebral ischemic tolerance is unclear. This study investigates the role of N-myc downstream–regulated gene-2 (NDRG2) in the neuroprotection of sevoflurane preconditioning in ischemic model both *in vivo* and *in vitro*.

Methods: At 2 h after sevoflurane (2%) preconditioning for 1 h, rats were subjected to middle cerebral artery occlusion for 120 min. Neurobehavioral scores (n = 10), infarct volumes (n = 10), cellular apoptosis (n = 6), and NDRG2 expression (n = 6) were determined at 24 h after reperfusion. *In vitro*, cultural astrocytes were exposed to oxygen–glucose deprivation for 4 h. Cellular viability, cytotoxicity, apoptosis, and NDRG2 expression (n = 6) were evaluated in the presence or absence of NDRG2-specific small interfering RNA or NDRG2 overexpression plasmid.

Results: Sevoflurane preconditioning decreased apoptosis (terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling–positive cells reduced to 31.2 ± 5.3% and cleaved Caspase-3 reduced to 1.42 ± 0.21 fold) and inhibited NDRG2 expression (1.28 ± 0.15 fold) and nuclear translocation (2.21 ± 0.29 fold) in ischemic penumbra. Similar effects were observed in cultural astrocytes exposed to oxygen–glucose deprivation. NDRG2 knockdown by small interfering RNA attenuated oxygen–glucose deprivation–induced injury (cell viability increased to 80.5 ± 4.1%; lactate dehydrogenase release reduced to 30.5 ± 4.0%) and cellular apoptosis (cleaved Caspase-3 reduced to 1.55 ± 0.21 fold; terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling–positive cells reduced to 18.2 ± 4.3%), whereas NDRG2 overexpression reversed the protective effects of sevoflurane preconditioning. All the data are presented as mean ± SD.

Conclusion: Sevoflurane preconditioning inhibits NDRG2 up-regulation and nuclear translocation in astrocytes to induce cerebral ischemic tolerance *via* antiapoptosis, which represents one new mechanism of sevoflurane preconditioning and provides a novel target for neuroprotection. **(Anesthesiology 2014; 121:549-62)**

SEVOFLURANE is a widely used inhalational anesthetic in clinical practice. Accumulating preclinical evidences have demonstrated that preconditioning with volatile anesthetics, such as sevoflurane, could induce ischemic tolerance both *in vitro* and *in vivo*.[1,2] The mechanisms underlying sevoflurane preconditioning were associated with inhibition of apoptosis,[3] activation of mitochondrial adenosine triphosphate–sensitive potassium channels,[4] reduction of excitatory amino acids,[5] and many more. However, the mechanism is not fully understood.

As the most abundant cells in central nervous system, astrocytes have crucial roles in regulating neuronal death and survival after cerebral ischemia.[6] Thus, astrocytes may be the potential targets for ischemic tolerance induced by precondition strategy. Previous studies showed that sevoflurane could increase the glutamate uptake of astrocytes under physiological circumstance[7] and delay the activation of astrocytes under different pathological conditions.[8,9] In this study, we focused on role of the indispensible astrocytes in the neuroprotective effect induced by sevoflurane preconditioning.

What We Already Know about This Topic

- The mechanisms of anesthetic-induced preconditioning against cerebral ischemia remain unclear but are crucial to optimizing neuroprotection strategies
- N-myc downstream–regulated gene-2 is an astrocytic protein implicated in ischemia-induced neuronal apoptosis

What This Article Tells Us That Is New

- Using *in vivo* and *in vitro* models of ischemia, sevoflurane preconditioning reduced astrocytic N-myc downstream–regulated gene-2 (NDRG2) expression and neuronal apoptosis, which was counteracted by NRDG2 overexpression
- Reduction of astrocytic NDRG2 expression by sevoflurane preconditioning is a novel astrocyte-mediated mechanism for anesthetic neuroprotection

N-myc downstream–regulated gene-2 (NDRG2) is one of the special genes that mainly expresses and plays its function in astrocyte rather than neuron of the adult brain.[10]

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Several studies demonstrated that NDRG2 is associated with astroglial activation,[11] brain tumor,[12] Alzheimer disease,[13] antidepression treatment,[14] and frontotemporal lobar degeneration,[15] suggesting its role in central nervous system and pathogenesis of neurobiological diseases. Our recent study first reported the spatial–temporal expression of NDRG2 in rat brain after transient focal cerebral ischemia, which indicates that alteration of NDRG2 in astrocytes might play an important role in cerebral ischemia.[16] Because NDRG2 is a downstream gene of p53-associated apoptosis pathway,[17] we hypothesize that sevoflurane preconditioning might induce astrocyte-specific neuroprotection *via* inhibiting the NDRG2-related apoptosis.

In this study, we try to elucidate the role of NDRG2 in sevoflurane preconditioning–induced ischemic tolerance in rats and cultured astrocytes. These are essential for understanding the mechanism of sevoflurane preconditioning to provide some new insights in ischemic tolerance and a novel target to develop neuroprotective strategy for cerebral ischemia.

Materials and Methods

Animals

All animal-related procedures were approved by the Ethics Committee for Animal Experimentation of Fourth Military Medical University (Xi'an, China) and preceded in accordance with the guidelines for Animal Experimentation of the University. The adult male Sprague–Dawley rats aged 8 to 12 weeks weighing 280 to 320 g and the neonatal Sprague–Dawley pups 1 to 3 days after birth were provided by the Experimental Animal Center of the Fourth Military Medical University.

Primary Astrocyte Culture

Primary astrocyte cultures were obtained from cerebral cortices of 1- to 3-day-old neonatal Sprague–Dawley pups. In brief, the brains were fetched, minced, and trypsinized (0.25% in phosphate-buffered saline) to cell suspension, which were then placed in Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA) with 20% (v/v) fetal bovine serum (Life Technologies), cultured in poly-L-lysine–coated (Sigma-Aldrich, St. Louis, MO) T75 flasks, and maintained at 37°C, under 90% relative humidity and 5% CO_2. The medium was half-replaced every 3 days. When cells reached confluence after 10 to 14 days, the flasks were shaken in 200 to 220 rpm for 14 to 16 h to remove microglia and oligodendrocytes. After shaking, more than 95% of the cultures were astrocytes determined by immunofluorescent staining with glial fibrillary acidic protein (GFAP). After isolation, the cells were subcultured into different plates according to different experiment.

Experiment Protocols

Experiment 1. To assess the effect of sevoflurane preconditioning on NDRG2 expression and localization *in vivo*, the animals were randomly divided into Sham, middle cerebral artery occlusion (MCAO), vehicle plus MCAO, and sevoflurane (sevoflurane preconditioning) plus MCAO groups. The rats in the Sham group underwent identical surgery without filament insertion, whereas the rats in the vehicle and sevoflurane groups were subjected to MCAO at 2 h after oxygen or sevoflurane preconditioning. Infarct volume, neurological function, activation of astrocytes, and expression and subcellular location of NDRG2 were evaluated at 24 h after reperfusion. Cellular apoptosis was detected by terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) and activation of Caspase-3 in ischemic penumbra.

Experiment 2. To further evaluate the role of NDRG2 in sevoflurane preconditioning *in vitro*, the cultural astrocytes were assigned to control, vector, NDRG2 oxygen–glucose deprivation (OGD), vehicle plus OGD, sevoflurane plus OGD, si-Control plus OGD, si-NDRG2 plus OGD, vector plus sevoflurane plus OGD, and NDRG2 plus sevoflurane plus OGD groups. The cells in the control group underwent identical medium replacement with normal culture medium instead of serum-free, glucose-free medium, whereas the cells in the vehicle and sevoflurane groups were subjected to OGD at 2 h after carrier gas or sevoflurane preconditioning. The small interfering RNAs (siRNAs) were transfected into the cultural astrocytes 24 h before OGD and the plasmids were transfected into the cells 24 h before sevoflurane preconditioning. Cell viability (methyl thiazolyl tetrazolium [MTT]), cytotoxicity (lactate dehydrogenase [LDH]), apoptosis (TUNEL and Caspase-3), and expression and subcellular location of NDRG2 were evaluated at 24 h after reoxygenation.

Sevoflurane Preconditioning

In Vivo. The protocol of sevoflurane preconditioning *in vivo* was performed as described previously.[2] An air-tight box ($50 \times 40 \times 30\ cm^3$) with gas inlet/outlet port was used. Rats were placed in the temperature-controlled transparent chamber for 1 h with 2.0% sevoflurane (sevoflurane preconditioning) flushing in 100% oxygen. Gas analyzer (MP-60; Phillips Medical Systems, Best, The Netherlands) monitored sevoflurane, oxygen, and carbon dioxide fraction of the inhaled and exhaled gas continuously. Soda lime (Molecular Products Limited, Essex, United Kingdom) was placed at the bottom of the container to clear the carbon dioxide.

In Vitro. The protocol of sevoflurane preconditioning *in vitro* was based on previous publication.[18] In brief, the cells were placed in an incubator chamber (Billups-Rothenberg, San Diego, CA), which was flushed for 5 min with 2.0% sevoflurane in the carrier gas of 95% air–5% CO_2, and then sealed at 37°C for 1 h (sevoflurane preconditioning). The concentration of sevoflurane in the chamber was determined by anesthetic gas analyzer.

Focal Cerebral Ischemia and Reperfusion

Transient focal cerebral ischemia was induced by MCAO for 120 min using an intraluminal filament technique as described in our previous studies[19] under pentobarbital sodium anesthesia (intraperitoneal injection, 40 mg/kg). The regional cerebral blood flow was monitored by a laser Doppler flowmetry (PeriFlux 5000; Perimed AB, Järfälla, Sweden). The MCAO was considered effective if the regional cerebral blood flow showed a sharp drop to less than 30% and recovered up to more than 80% of baseline (preischemia) level; animals that did not meet this requirement were excluded. Throughout the whole process of MCAO, the temporal temperature was maintained at 37° ± 1°C by a thermostatic blanket.

Oxygen–Glucose Deprivation

The OGD was performed as described previously.[20] In brief, the culture medium was replaced with serum- and glucose-free Dulbecco's Modified Eagle's Medium and placed in an incubator chamber, which was flushed for 5 min with 95% N_2 and 5% CO_2 and then sealed at 37°C (hypoxia). Control cultures were incubated for the same period of time at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After 4 h challenge, astrocytes were removed from the anaerobic chamber, and the medium was replaced with Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. The cells were maintained for further 24 h at 37°C in a humidified 5% CO_2 incubator to generate reperfusion insult.

Interference of NDRG2 in Cultural Astrocytes

The siRNA oligonucleotides were purchased from QIAGEN Company (Hilden, Germany). The sequences targeting at NDRG2 are 5'-GCAUCCUGCAGUACUUAAATT-3' and 5'-UUUAAGUACUGCAGGAUGCAA-3'. The siRNAs were transfected into cells 24 h before OGD with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

NDRG2 Overexpression Plasmid Construction

The plasmids were constructed according to previous research. In brief, DNA fragments encoding rat NDRG2 and human NDRG2 were polymerase chain reaction (PCR)–amplified with the following primers (forward and reverse): rat NDRG2, 5'-GAATTCTATGGCAGAGCTTCAGGAGGT-3' and 5'-GGATCCTCAACAGGAGACTTCCATGGT-3'; human NDRG2, 5'-ATGGCGGAGCTGCAGGAGGTGC-3' and 5'-TGAGGAACGAGGTCTGGGTGGG-3'. The DNA fragments were cloned into the *Eco*R/*Bam*HI sites of the pEGFP-C1 vector (Clontech, Palo Alto, CA). The expression vectors were transfected into cells 24 h before different treatments with Lipofectamine 2000 according to the manufacturer's instructions.

Neurobehavioral Evaluation and Infarct Assessment

The neurobehavioral evaluation and infarct assessment were performed as our previous studies.[19,21] In brief, at 24 h after reperfusion, the animals were neurologically assessed according to the method described in the study by Garcia *et al.*[22] by an investigator who was blinded to animal grouping. Then, the rats were decapitated under anesthesia, and 2-mm-thick coronal sections throughout brain were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) to evaluate the infarct volume. The infarct volume percentages were calculated by an investigator unaware of the experimental grouping with Swanson method.[23] After the 2,3,5-triphenyltetrazolium chloride staining, the brain slices were photographed with a digital camera (Kodak DC240; Eastman Kodak Co., Rochester, NY) connected to a computer. The unstained white area was defined as lesioned, and the stained red area was defined as nonlesioned. The nonlesioned area in right hemisphere (RN) and total area in left hemisphere (LT) were measured using the image analysis software (Adobe Photoshop CS5; Adobe Systems Incorporated, San Jose, CA) by an investigator blinded to the experimental grouping. The noninfarct volume of right hemisphere and total volume of left hemisphere were calculated: V_{RN} = RN × slice thickness (2 mm) and V_{LT} = LT × slice thickness (2 mm). The infarct volume was measured as follow: $\%V_I = 100 \times (V_{LT} - V_{RN})/V_{LT}$.

MTT Assay and LDH Release

Both the cell viability and cytotoxicity were investigated as our previous studies.[20] Cell viability was measured using the MTT assay. In brief, the primary astrocytes were seeded in 96-well plates and subjected to various treatments as described earlier. MTT (Sigma-Aldrich) solution (final concentration of 0.5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37°C. After the medium was carefully removed, the blue-colored formazan product was dissolved with dimethyl sulfoxide (Sigma-Aldrich) and the absorbance was subsequently measured at 570 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA). Cell survival rates were expressed in percentage of the value of cells in control group.

Cytotoxicity was determined by the release of LDH, a cytoplasmic enzyme released from cells, and a marker of membrane integrity. LDH release into the culture medium was detected using a diagnostic kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The activity unit was defined as units per deciliter.

Immunofluorescent Staining

At 24 h after reperfusion, the rats were deeply anesthetized and transcardially perfused with saline and 4% paraformaldehyde, and 1-μm-thick paraffin sections were prepared with coronal brain tissue −3.0 to −5.0 mm from Bregma (covering the infarct area and ischemic penumbra according

to Ashwal protocol).[24] The apoptotic cells in the ischemic penumbra were detected by TUNEL staining (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Then, the TUNEL-positive cells and 4',6-diamidino-2-phenylindole (Sigma-Aldrich)–positive cells were counted from images by a blinded investigator, acquired with a 40× objective from three random areas in penumbra, and expressed as TUNEL-positive cells per 4',6-diamidino-2-phenylindole–positive cells. The cultural astrocytes were seeded onto L-lysine–coated slides and processed in the same way.

For double-labeling immunofluorescent staining, sections and astrocytes-seeded slides were incubated with mouse anti-GFAP antibody (1:1,000; Abcam, Cambridge, United Kingdom) and rabbit anti-NDRG2 antibody (1:200; Abcam) overnight at 4°C. After washing three times with phosphate-buffered saline, sections and astrocytes slides were incubated with Alexa-594–conjugated goat-anti-mouse and Alexa 488 goat-anti-rabbit secondary antibody (1:400 for both; Life Technologies) for 2 h in room temperature. At last, the sections and slides were dyed with 4',6-diamidino-2-phenylindole for nuclear staining. The staining results were observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and the images were captured.

Western Blot

In vivo, at 24 h after reperfusion, the ischemic penumbras were microdissected according to established protocols in rodent models of unilateral proximal MCAO.[24] The tissues were homogenized in RIPA lysis buffer (Beyotime, Nantong, China) with ×1 Roche complete protease inhibitor cocktail (Roche Diagnostics) and 1 mM phenylmethylsulfonylfluoride (Beyotime), and Western blot was performed using following primary antibodies: rabbit anti-NDRG2 antibody (1:2,000; Abcam), rabbit anti-cleaved Caspase-3 antibody (1:500; Abcam), and mouse anti-β-actin monoclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse antibody (1:5,000; Pierce, Rockford, IL) was used. *In vitro*, at 24 h after reoxygenation, the cultured astrocytes were collected and disposed as *in vivo*.

To investigate the nuclear/cytoplasm distribution of NDRG2, the nuclear and cytoplasmic protein of the ischemic penumbra and cultural astrocytes were extracted according to the manufacture's protocol (NE-PER nuclear and cytoplasmic extraction reagents; Pierce). All extracts were analyzed by Western blot likewise.

Real-time PCR

The ischemic penumbra tissues for real-time PCR analysis were dissected in the same way as Western blot. The total RNA was extracted with Trizol (Life Technologies). After equalization of the RNA quantity in each group, the reverse-transcription and real-time PCR was performed using commercially available kit (TaKaRa, Dalian, China). Quantitative PCR was completed with the Bio-Rad iQ5 Gradient Real-Time PCR system (Bio-Rad Laboratories and Life Technologies). The primers used for rat NDRG2 and β-actin (control) were as follows (forward and reverse): NDRG2, 5'-AACTTTGAGCGAGGTGGTGAGA-3' and 5'-ATTCCACCACGGCATCTTCA-3'; β-actin, 5'-TTGCTGACAGGATGCAGAAGG-3' and 5'-ATTGGTGGCTCTATCCTGGC-3'. *In vitro*, at 24 h after reoxygenation, the cultured astrocytes were collected and disposed as *in vivo*.

Statistical Analysis

All the experiments were performed a minimum of three times. SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analyses. All values, except for neurologic scores, were expressed as mean ± SD. Significant differences between experiments were assessed by one-way ANOVA followed by Tukey *post hoc* test. The neurologic scores, presented as median (interquartile range), were analyzed by Kruskal–Wallis test followed by the Mann–Whitney U test with Bonferroni correction. Two-tailed *P* values of less than 0.05 were considered to be statistically significant.

Results

Sevoflurane Preconditioning Attenuated Cerebral Ischemic Injuries in Rats

As shown in figure 1, sevoflurane preconditioning significantly improved the neurological scores (12.75 [11.38 to 13.50]) and reduced the infarction volumes (25.7 ± 5.7%) compared with those of the vehicle plus MCAO group (7.00 [6.50 to 8.50], 48.3 ± 8.9%, $P < 0.05$). No statistical significance of neurological scores or infarction volumes was detectable between MCAO and vehicle plus MCAO groups.

As shown in figure 2A, positive TUNEL staining was hardly detected in brain sections of sham animals at 24 h after reperfusion, whereas large numbers of TUNEL-positive cells were seen in the ischemic penumbra in the vehicle plus MCAO group. In contrast, only a small amount of TUNEL staining was observed in the sevoflurane group. Quantitative analysis showed that sevoflurane preconditioning significantly reduced the number of TUNEL-positive cells (31.2 ± 5.4%, $P < 0.05$) compared with the MCAO (62.5 ± 8.5%) and vehicle plus MCAO (61.5 ± 9.2%) groups. No statistical difference was found between the MCAO and vehicle plus MCAO groups (fig. 2B). Western blot of the ischemic penumbra tissue for cleaved Caspase-3 showed the same result (fig. 2C).

Sevoflurane Preconditioning Inhibited Astrocyte Activation, NDRG2 Up-regulation, and Nuclear Translocation in Astrocytes after Reperfusion in Rats

After reperfusion, the number of activated astrocytes was increased, as shown by the characteristic morphology, number, and appearance of GFAP-positive staining. Furthermore, Western blot also showed that the expression of GFAP in ischemic penumbra was increased after reperfusion (vehicle plus MCAO, 2.05 ± 0.23 fold). Sevoflurane preconditioning inhibited the

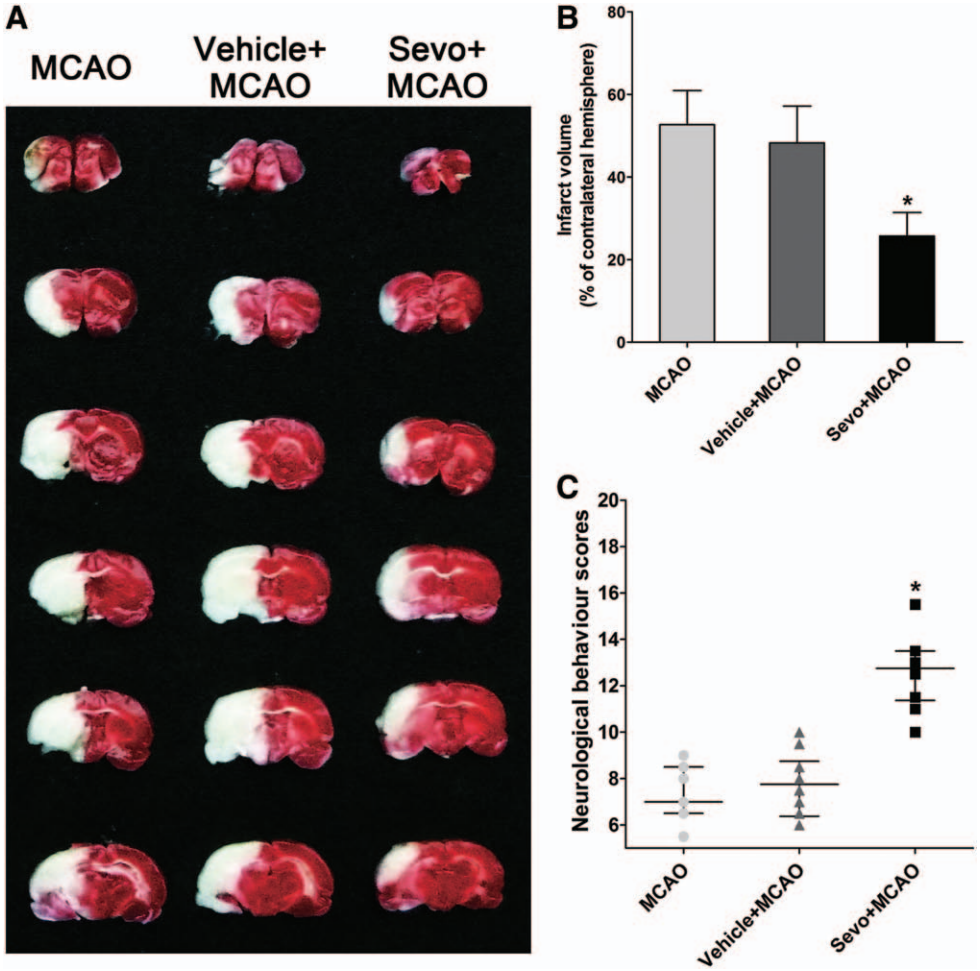

Fig. 1. Infarct volume percentage and neurological behavior score of rats subjected to middle cerebral artery occlusion (MCAO). (*A*) Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in comparable sections of rat brain from three groups at 24 h after reperfusion. Sevoflurane preconditioning (Sevo) reduced brain infarct volumes (*B*) and improved neurological scores (*C*). Infarct volume percentages are expressed as mean ± SD (n = 10); neurological behavior scores are represented as median (interquartile range) with the individual values (n = 10). *$P < 0.05$ in comparison with vehicle group.

astrocyte activation evidenced by reduction of the number of GFAP-positive astrocytes and the expression of GFAP in ischemic penumbra (sevoflurane, 1.35 ± 0.21 fold) (fig. 3).

As shown in figure 4, A and B, the NDRG2 expression in ischemic penumbra was up-regulated after transient focal cerebral ischemia (vehicle plus MCAO, NDRG2 messenger RNA [mRNA], 3.75 ± 0.35 fold; NDRG2 protein, 1.95 ± 0.18 fold). However, the expressions of NDRG2 mRNA (2.21 ± 0.28 fold, $P < 0.05$) and protein (1.28 ± 0.15 fold, $P < 0.05$) in sevoflurane group at 24 h after reperfusion were decreased as compared with MCAO and vehicle plus MCAO group, whereas there was no difference between MCAO group and vehicle plus MCAO group.

Immunofluorescent staining showed that NDRG2 signals were mainly colocalized with GFAP-positive astrocytes. The expression of NDRG2 in nucleus was increased after reperfusion, whereas the level of NDRG2 in cytoplasm was relatively decreased. Sevoflurane preconditioning reduced the expression of NDRG2 protein expression in astrocytes and inhibited translocation of NDRG2 from cytoplasm to nucleus after reperfusion (fig. 4, C and D).

To further support the assumption of nuclear/cytoplasm location shift of NDRG2 after sevoflurane preconditioning and MCAO, we extracted the nuclear and cytoplasmic protein of the ischemic penumbra separately and used Western blot to find the change of NDRG2 expression in both parts. As shown in figure 4E, the ratio of nuclear NDRG2 and cytoplasmic NDRG2 was increased in ischemic penumbra (vehicle plus MCAO, 4.30 ± 0.55 fold) and sevoflurane preconditioning reversed this increase of nuclear NDRG2/cytoplasmic NDRG2 ratio (2.21 ± 0.29 fold, $P < 0.05$), which was in consistent with previous morphological changes.

Sevoflurane Preconditioning Protects Cultured Astrocytes against OGD-induced Injury

For that NDRG2 mainly expresses in astrocytes of the central nervous system, we introduced primary astrocyte culture

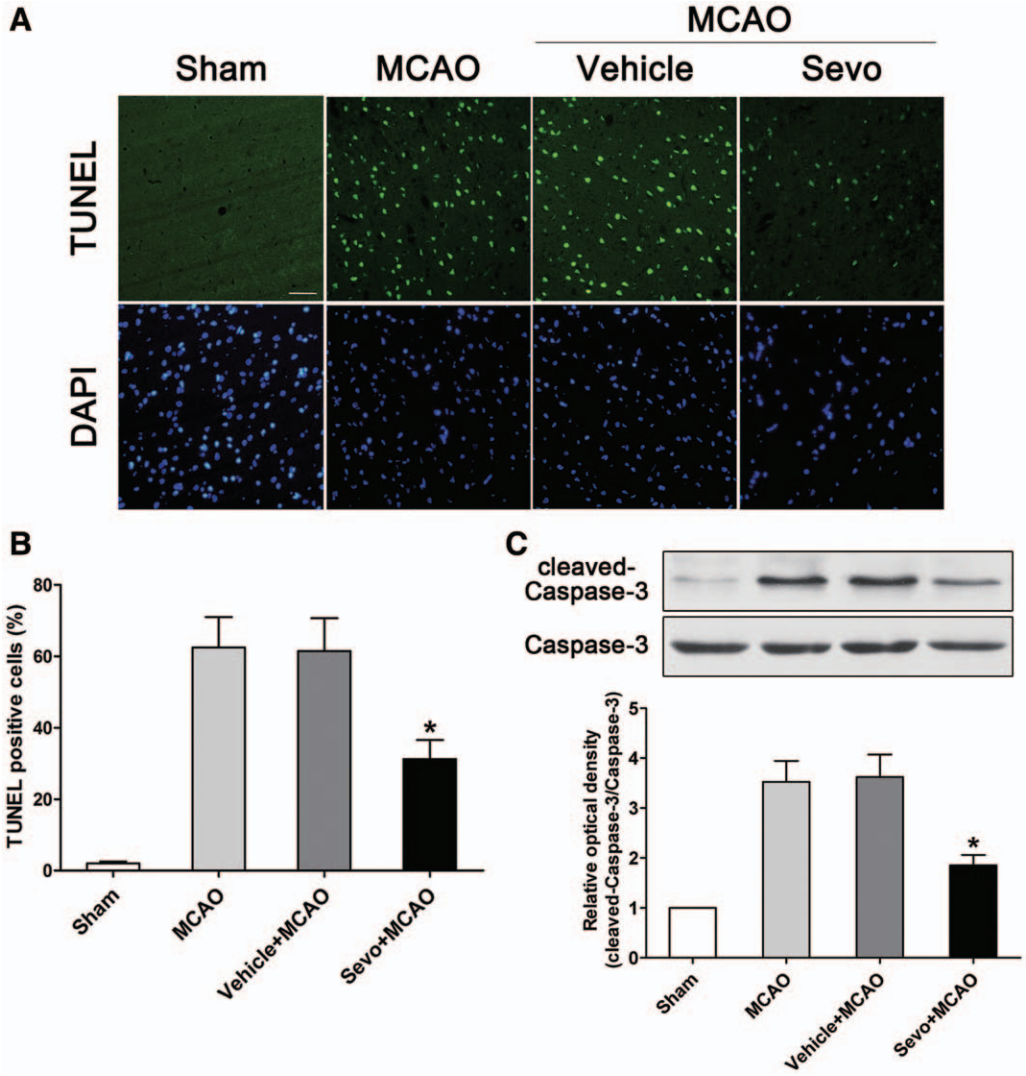

Fig. 2. Terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining and cleaved Caspase-3 expression in ischemic penumbra of rats subjected to middle cerebral artery occlusion (MCAO). Sevoflurane preconditioning (Sevo) attenuated positive TUNEL staining (*A*, *B*) and the high-level expression of cleaved Caspase-3 (*C*) in the ischemic penumbra. *Bar* = 100 μm in *A*. Data are expressed as mean ± SD (n = 6). **P* < 0.05 in comparison with vehicle group. DAPI = 4',6-diamidino-2-phenylindole.

and OGD model to further evaluate the role of NDRG2 in sevoflurane preconditioning. Exposure of primary cultured astrocytes to OGD resulted in marked LDH release (vehicle plus OGD, 62.5 ± 6.2%) and MTT reduction (vehicle plus OGD, 45.2 ± 3.5%) in comparison with control cultures. Sevoflurane preconditioning significantly inhibited the OGD-induced increase in LDH release (32.5 ± 4.0%, $P < 0.05$) and decrease in MTT (78.5 ± 4.0%, $P < 0.05$) reduction (fig. 5).

In addition, at 24 h after the reoxygenation, the sevoflurane preconditioning also significantly inhibited OGD-induced cellular apoptosis evaluated by TUNEL-positive cells (24.2 ± 4.4%, $P < 0.05$) and the expression level of cleaved Caspase-3 (1.42 ± 0.21 fold, $P < 0.05$) compared with the OGD and vehicle plus OGD groups (vehicle plus OGD, 45.5 ± 7.2%, 2.35 ± 0.24 fold) (fig. 6). There was no statistical difference between OGD and vehicle plus OGD groups.

Sevoflurane Preconditioning Inhibited NDRG2 Up-regulation and Nuclear Translocation in Cultured Astrocytes Exposed to OGD

Consistent with results *in vivo*, the expressions of NDRG2 mRNA and protein in sevoflurane plus OGD group (NDRG2 mRNA, 1.82 ± 0.25 fold, $P < 0.05$; NDRG2 protein, 1.25 ± 0.19 fold, $P < 0.05$) at 24 h after reoxygenation were decreased in comparison with vehicle plus OGD group (NDRG2 mRNA, 4.13 ± 0.45 fold; NDRG2 protein, 2.10 ± 0.23 fold), whereas no difference between OGD group and vehicle plus OGD group was detected (fig. 7, A and B). Furthermore, sevoflurane preconditioning inhibited the up-regulation of NDRG2 protein expression and the

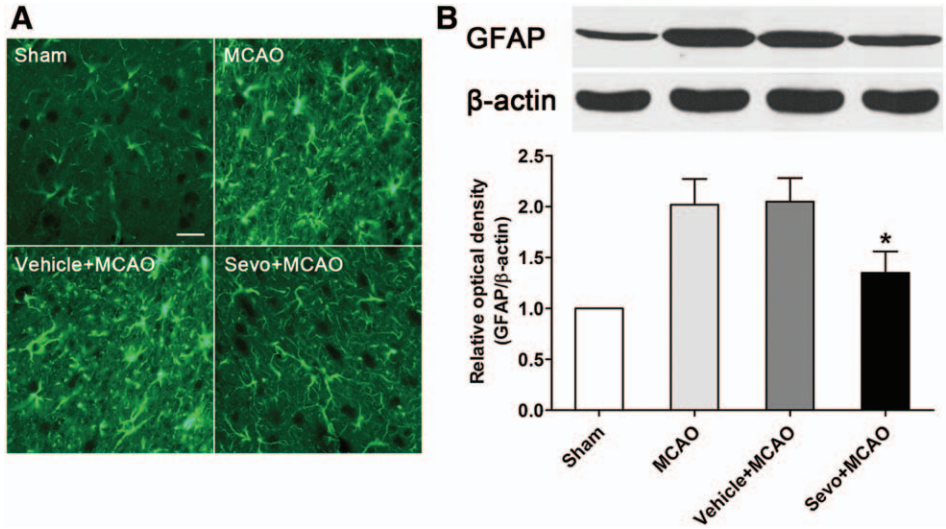

Fig. 3. Astrocyte activation in ischemic penumbra of rats subjected to middle cerebral artery occlusion (MCAO). Morphological change of astrocytes (*A*) and expression of glial fibrillary acidic protein (GFAP) (*B*) in the ischemic penumbra were inhibited by sevoflurane preconditioning (Sevo). *Bar* = 40 μm in *A*. Data are expressed as mean ± SD (n = 6). **P* < 0.05 in comparison with vehicle group.

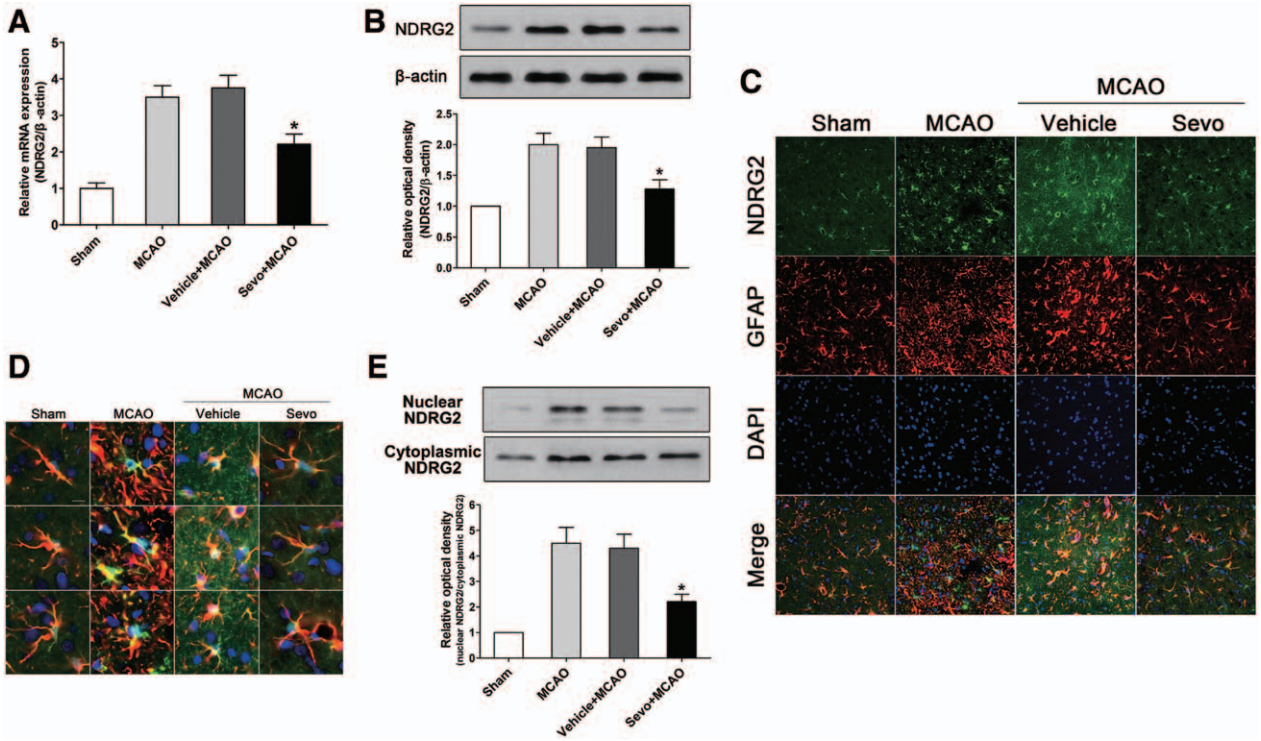

Fig. 4. Expression of N-myc downstream–regulated gene-2 (NDRG2) messenger RNA (mRNA) and protein in the ischemic penumbra of rats subjected to middle cerebral artery occlusion (MCAO). Sevoflurane preconditioning (Sevo) inhibited ischemia–reperfusion-induced NDRG2 up-regulation (*A*, *B*), nuclear translocation (*C*, *D*), and increase of nuclear NDRG2 expression (*E*) in ischemic penumbra. *Bar* = 100 μm in *C* and *bar* = 20 μm in *D*. Data are expressed as mean ± SD (n = 6). **P* < 0.05 in comparison with vehicle group. DAPI = 4',6-diamidino-2-phenylindole; GFAP = glial fibrillary acidic protein.

translocation of NDRG2 from cytoplasm to nucleus after reoxygenation in cultured astrocytes (vehicle plus OGD *vs.* sevoflurane plus OGD, 3.75 ± 0.49 fold *vs.* 1.78 ± 0.23, *P* < 0.05), which was proved by both double-labeling immunofluorescent and cell fraction assay (fig. 7, C and D).

NDRG2 Knockdown by siRNA Attenuates Cellular Injury and Apoptosis in Cultured Astrocytes Exposed to OGD

Because the expression of NDRG2 was significantly higher in astrocytes exposed to OGD than in the Sham cells, we tried to knockdown NDRG2 by siRNA to find out the role of NDRG2

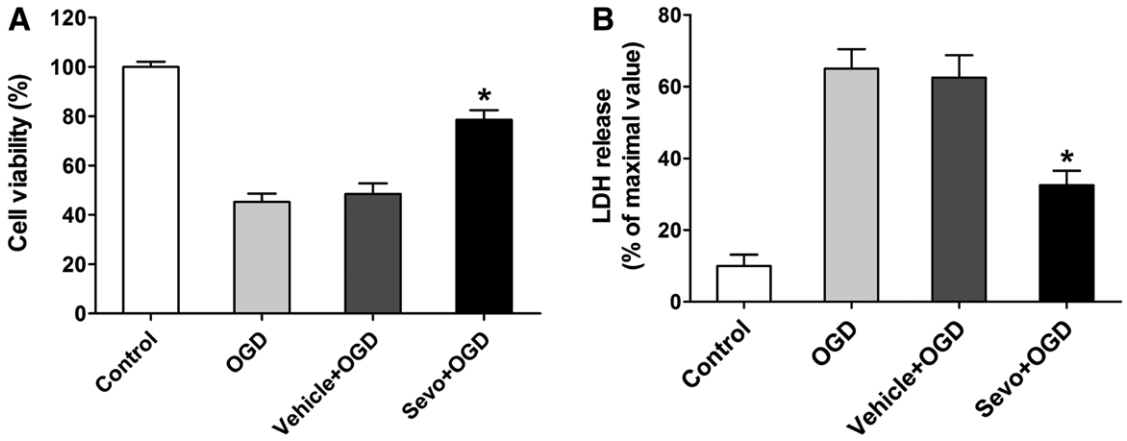

Fig. 5. Methyl thiazolyl tetrazolium and lactate dehydrogenase (LDH) release assay in cultural astrocytes exposed to oxygen–glucose deprivation (OGD). Cell viability of astrocytes was increased (*A*), whereas cytotoxicity was inhibited (*B*) by sevoflurane preconditioning (Sevo). Data are expressed as mean ± SD (n = 6). *$P < 0.05$ in comparison with vehicle group.

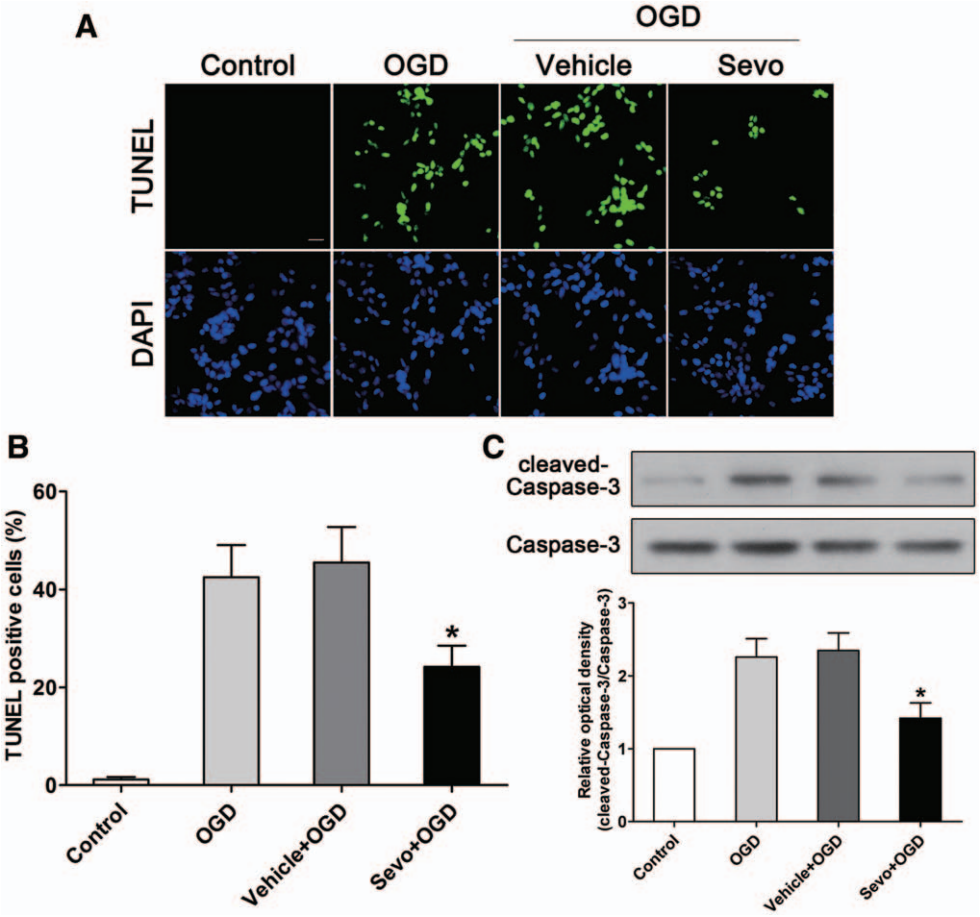

Fig. 6. Terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining and cleaved Caspase-3 expression in cultural astrocytes exposed to oxygen–glucose deprivation (OGD). Sevoflurane preconditioning (Sevo) attenuated positive TUNEL staining (*A*, *B*) and high-level expression of cleaved Caspase-3 (*C*) in cultural astrocytes. *Bar* = 40 μm in *A*. Data are expressed as mean ± SD (n = 6). *$P < 0.05$ in comparison with vehicle group. DAPI = 4',6-diamidino-2-phenylindole.

in OGD-induced cellular injury and apoptosis. The results were similar to our previous work in interleukin-6–differentiated C6 glioma cells. Both the OGD-induced injury and cellular apoptosis were alleviated in the NDRG2 siRNA group.

The cell viability assessed by MTT assay of the cultured astrocytes in NDRG2 siRNA (si-NDRG2) plus OGD group (80.5 ± 4.1%) was higher than that of control siRNA with scrambled sequence (si-Con) plus OGD group (44.5 ± 4.3%,

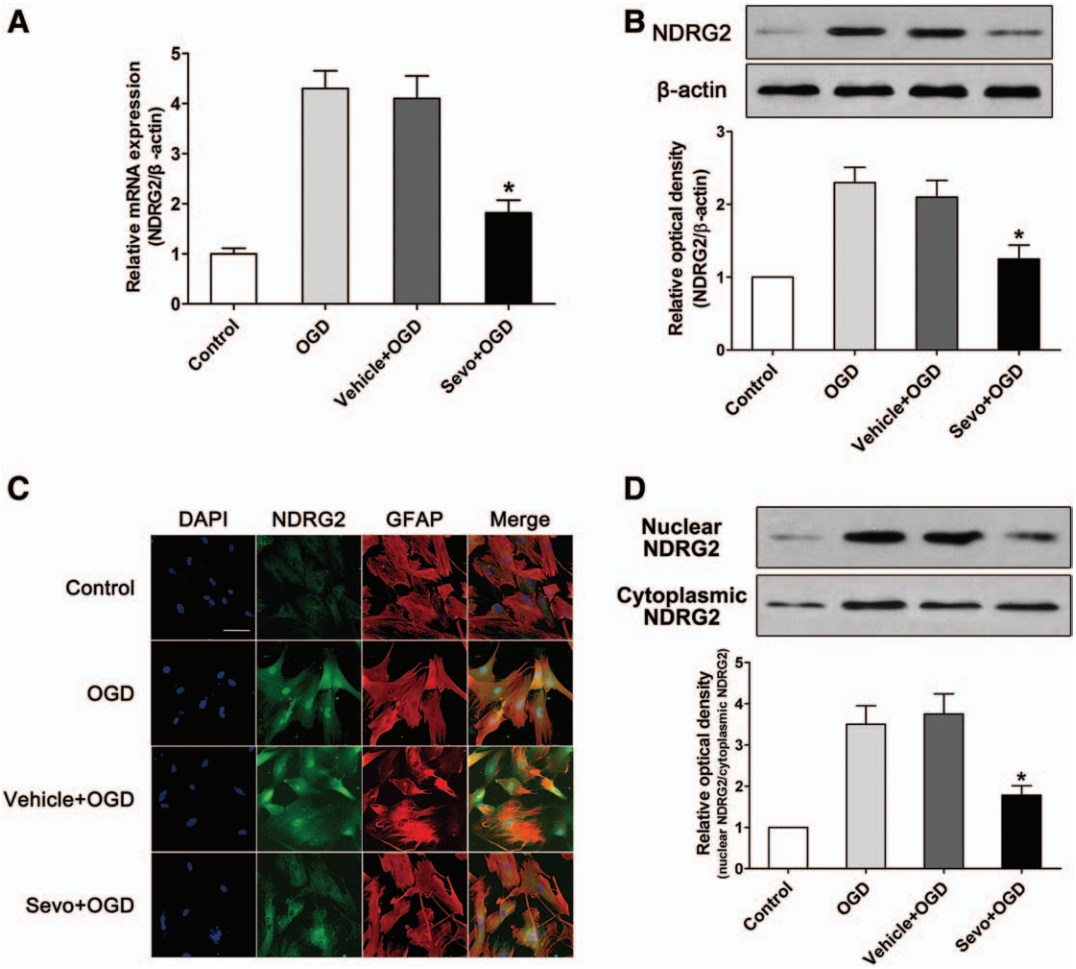

Fig. 7. N-myc downstream–regulated gene-2 (NDRG2) messenger RNA (mRNA) and protein expression in cultural astrocytes exposed to oxygen–glucose deprivation (OGD). Sevoflurane preconditioning (Sevo) inhibited anoxia/reoxygenation induced NDRG2 up-regulation (*A*, *B*), nuclear translocation (*C*), and increase of nuclear NDRG2 expression (*D*) in the cultural astrocytes. *Bar* = 60 μm in *C*. Data are expressed as mean ± SD (n = 6). *$P < 0.05$ in comparison with vehicle group. DAPI = 4',6-diamidino-2-phenylindole; GFAP = glial fibrillary acidic protein.

$P < 0.05$), whereas the LDH release assay measured cellular injury of the astrocytes in si-NDRG2 plus OGD (30.4 ± 4.0%) was decreased compared with si-Con plus OGD (59.3 ± 6.2%, $P < 0.05$) at 24 h after reoxygenation. Western blot showed that the si-NDRG2 significantly down-regulated the expression of NDRG2 (0.42 ± 0.08 fold, $P < 0.05$) and cleaved Caspase-3 (1.55 ± 0.21 fold, $P < 0.05$) compared with either sham or si-Con (NDRG2, 2.41 ± 0.27 fold; cleaved Caspase-3, 2.52 ± 0.29 fold) at 24 h after reoxygenation. TUNEL staining demonstrated that TUNEL-positive astrocytes were decreased in si-NDRG2 plus OGD group (18.2 ± 4.3%, $P < 0.05$) compared with si-Con plus OGD (44.5 ± 6.5%) at 24 h after reoxygenation. These results indicated that silencing NDRG2 by siRNA had a protective effect against OGD-induced injury *via* inhibition of cellular apoptosis in cultural primary astrocytes (fig. 8). In contrast, cotransfection of astrocytes with si-NDRG2 and human NDRG2 expression construct that served as an si-NDRG2–resistant construct, which restores NDRG2 expression level, reversed the NDRG2 knockdown–induced reductions in astrocytes injury and apoptosis (fig. 8).

NDRG2 Overexpression Reverses the Cytoprotection of Sevoflurane Preconditioning against OGD in Cultured Astrocytes

Because the knockdown of NDRG2 by siRNA could attenuate OGD-induced injury and cellular apoptosis in cultural primary astrocytes, we constructed overexpression system of NDRG2 by cloning the NDRG2-encoded DNA fragment onto a pEGFP-C1 vector (NDRG2 vector) and transfected the vector into the astrocytes before sevoflurane preconditioning to investigate the influence of NDRG2 overexpression on the protective effect of sevoflurane preconditioning.

The overexpression of NDRG2 was verified by Western blot at 24 h after the administration. The result showed that the pEGFP-C1 constructs expressing NDRG2 (NDRG2 vector) significantly up-regulated the expression of NDRG2 (6.25 ± 0.68 fold, $P < 0.05$) compared with Sham

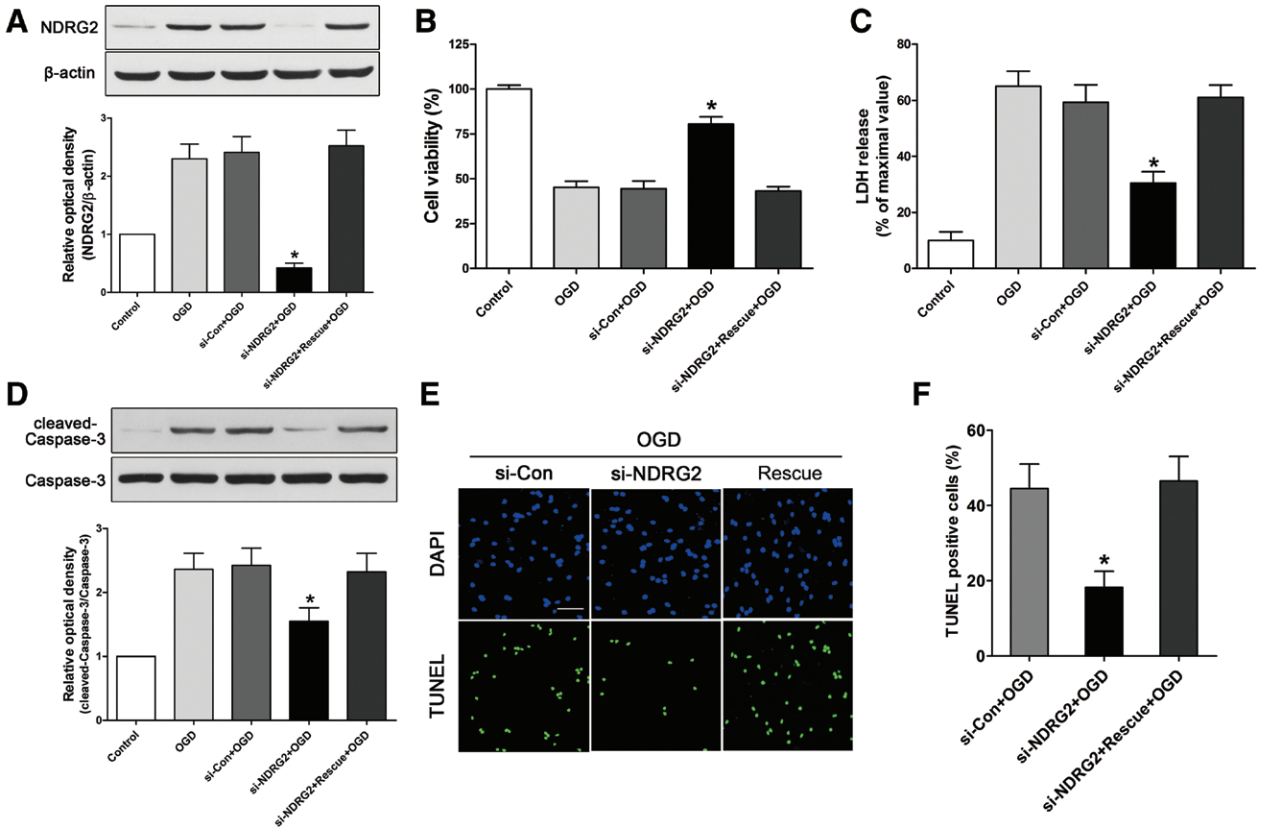

Fig. 8. Effect of N-myc downstream–regulated gene-2 (NDRG2) down-regulation on cellular injury in cultural astrocytes exposed to oxygen–glucose deprivation (OGD). NDRG2-specific small interfering RNA significantly inhibited the expression of NDRG2 in cultural astrocytes (*A*). Down-regulation of NDRG2 increased cell viability (*B*) of astrocytes, whereas decreased cytotoxicity (*C*) and apoptosis which was tested by cleaved Caspase-3 expression (*D*) and positive terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining (*E*, *F*) of astrocytes after OGD. These effects were reversed by coexpression of si-NDRG2–resistant construct (Rescue) (*A–F*). *Bar* = 80 μm in *E*. Data are expressed as mean ± SD (n = 6). **P* < 0.05 in comparison with si-Con group. DAPI = 4',6-diamidino-2-phenylindole; LDH = lactate dehydrogenase; si-Con = control small interfering RNA with scramble sequence.

or pEGFP-C1 (vector) (1.08 ± 0.22 fold). As compared with astrocytes transfected with vector, the cell viability (28.5 ± 2.3%, $P < 0.05$) was decreased, whereas the LDH release (78.6 ± 2.3%, $P < 0.05$) was increased in astrocytes transfected with NDRG2 vector at 24 h after reoxygenation. Furthermore, the increase of Caspase-3 cleavage (3.82 ± 0.43 fold, $P < 0.05$) was also observed in NDRG2 vector group (fig. 9, A–D).

At 24 h after reoxygenation, the cell viability of the cultural astrocytes in sevoflurane plus NDRG vector group was lower (47.5 ± 3.1%, $P < 0.05$), whereas the LDH release was higher (65.6 ± 6.5%, $P < 0.05$) compared with sevoflurane plus vector group. Both the TUNEL-positive staining (77.5 ± 9.5%, $P < 0.05$) and the expression of cleaved Caspase-3 (2.35 ± 0.29 fold, $P < 0.05$) were increased in sevoflurane plus NDRG2 vector group compared with sevoflurane plus vector group at 24 h after reoxygenation, indicating that the protective effect of sevoflurane preconditioning against OGD-induced cell injury and apoptosis was significantly reversed by the overexpression of NDRG2 (fig. 9, E–I).

Discussion

Protective effects of inhalational anesthetics preconditioning were first reported in both *in vitro* and *in vivo* models of myocardial ischemia.[25,26] Recent studies have shown that inhalational anesthetics preconditioning exerts neuroprotective effects against cerebral ischemia *in vitro* and *in vivo*.[27–32] As one of inhalational anesthetics, sevoflurane has been investigated in various models by using both immediate and delayed preconditioning paradigm.[2,21,33] These studies showed that sevoflurane preconditioning could induce neuroprotective effects by reduction in cellular injury and inhibition of neuronal apoptosis.[5,12] Our team has accomplished a lot of work on repetitive sevoflurane preconditioning, which needs 5 consecutive days of sevoflurane preconditioning before ischemia. In the current study, we first verified whether single preconditioning with sevoflurane would still exert neuroprotective effect against focal cerebral ischemia. Just like the effects of repetitive sevoflurane preconditioning on cerebral ischemia, we found that single preconditioning with sevoflurane attenuated ischemia–reperfusion injury through reduction

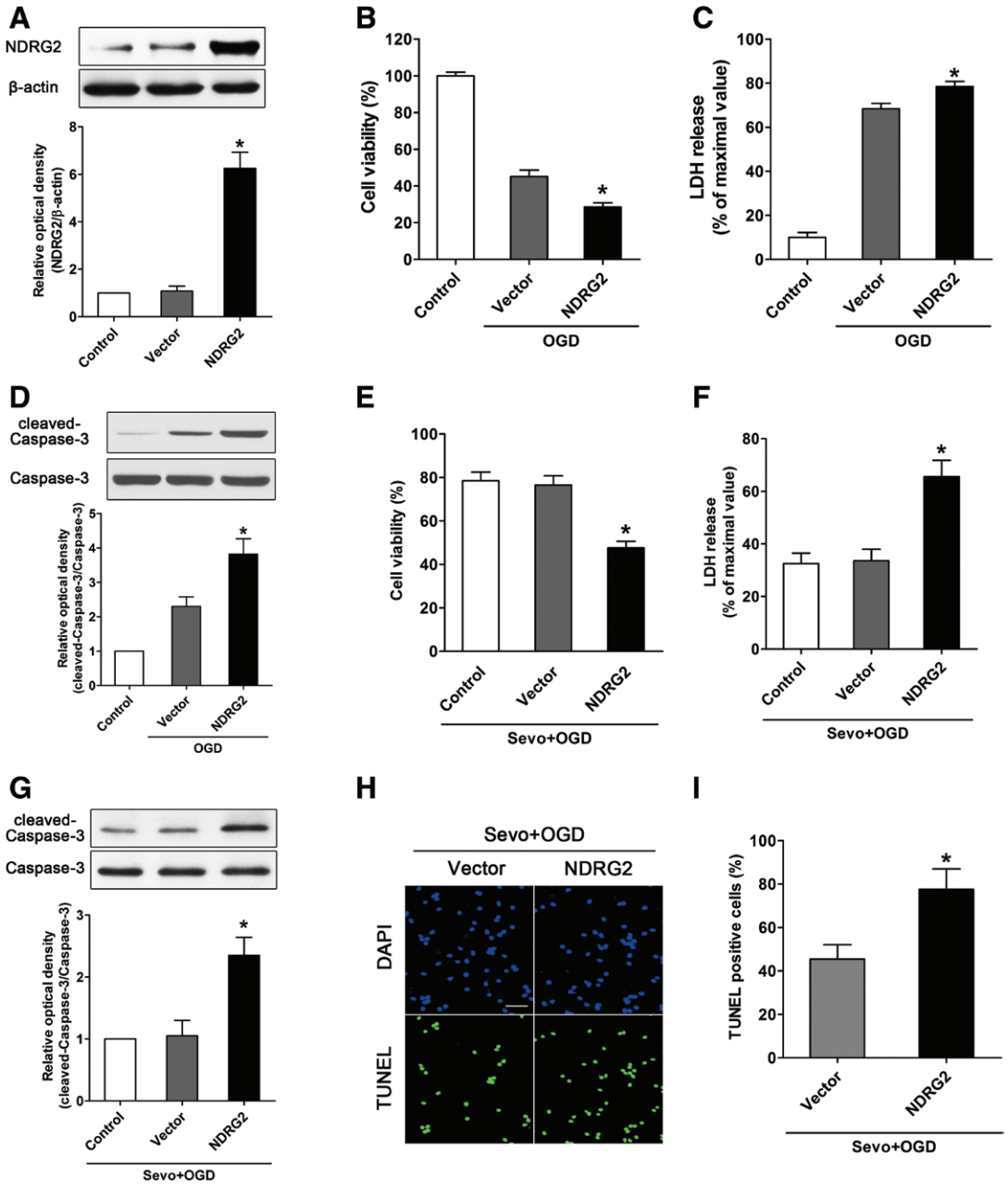

Fig. 9. Effects of N-myc downstream–regulated gene-2 (NDRG2) up-regulation on cytoprotection of sevoflurane preconditioning (Sevo) exposed to oxygen–glucose deprivation (OGD). NDRG2-pEGFP-C1 vector significantly increased the expression of NDRG2 in cultural astrocytes (*A*). Overexpression of NDRG2 aggravated OGD-induced reduction of cell viability (*B*) and increase of cytotoxicity (*C*) and apoptosis by cleavage of Caspase-3 (*D*). Up-regulation of NDRG2 attenuated sevoflurane preconditioning–induced increase of cell viability (*E*), reduction of cytotoxicity (*F*), and inhibition of apoptosis by cleaved Caspase-3 expression (*G*) and positive terminal deoxynucleotidyl transferase–mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining (*H*, *I*). *Bar* = 80 μm in *H*. Data are expressed as mean ± SD (n = 6). **P* < 0.05 in comparison with vector group. DAPI = 4',6-diamidino-2-phenylindole; LDH = lactate dehydrogenase; Vector = empty pEGFP-C1 vector.

of infarction size, improvement of neurological function, and inhibition of apoptosis. These evidences confirmed the neuroprotective effects of single sevoflurane preconditioning against brain ischemia, but cellular mechanisms underlying this neuroprotection might be more complicated.

Because of the essential role of neurons in brain function, attention on cerebral ischemia had been mainly focused on neuronal injury and its potential mechanisms. Although the number of astrocytes is more than five-fold of neurons in brain, their fate after injury had been neglected in the past studies. With a revolution of understanding about physiology and pathology of astrocytes,[34] astrocytes have been implicated to perform various functions including formation of blood–brain barrier, regulation of blood flow,[35] control of energy

metabolism,[36] mediation of synaptic transmission,[37] and maintenance of the homeostasis in synaptic interstitial fluid,[38,39] rather than just structurally connect neurons. Therefore, investigation on astrocyte-specific function might provide novel targets to develop neuroprotective strategy for cerebral ischemia.

To date, few studies had paid attention to the role of astrocytes in sevoflurane preconditioning–induced neuroprotection. In the current study, GFAP-positive staining showed that astrocyte morphology change was restored by sevoflurane preconditioning. In the matter of fact, astrocyte morphology change is a critical response at the early phase after ischemia and leads to brain edema and a rapid increase of intracerebral pressure.[40] In addition, repetitive sevoflurane preconditioning could protect the integrity of blood–brain barrier, which is associated with suppression of astrocyte activation.[41] Here, the cerebral ischemia-induced activation of astrocytes appeared to have been inhibited by single sevoflurane preconditioning. Furthermore, activated astrocytes produce the proinflammatory factors, which is detrimental to recovery after cerebral ischemia.[42] In the previously published studies of inhalational anesthetics preconditioning, sevoflurane preconditioning suppresses the activation of inflammation-related signaling pathways and expression of inflammatory cytokines,[43] demonstrating that sevoflurane preconditioning probably had antiinflammatory effects against ischemia. Further *in vitro* experiments showed that sevoflurane preconditioning enhanced the tolerance of primary astrocytes against OGD and inhibited apoptosis of astrocytes. Thus, neuroprotection of sevoflurane preconditioning might be tightly relevant to its regulation of astrocyte functions.

Although many studies have shown that sevoflurane preconditioning conferred neuroprotective effects *via* regulation of various intracellular signaling pathways, it is unclear whether sevoflurane preconditioning could regulate some astrocyte-specific signaling pathways. As a member of NDRG family, NDRG2 is predominant expressed in astrocytes of the central nervous system. Although NDRG2 has been suggested to act as a tumor suppressor and is involved in cell proliferation and differentiation, its role in regulating astrocytic function is also reported in many studies.[14,16,44] In respect of cerebral ischemia, our previous study has demonstrated that expression of NDRG2 was up-regulated after reperfusion. NDRG2 was mainly expressed in astrocytes and colocalized with apoptotic cell, suggesting up-regulation of NDRG2 might be associated with apoptosis of astrocytes after cerebral ischemia.[16] In our recent study, we revealed that OGD also led to the similar alteration of NDRG2 in astrocyte originated from C6 glioma cells.[17] Sevoflurane preconditioning decreased the ischemia-induced enhancement of NDRG2 expression and inhibited the nuclear translocation of NDRG2 in ischemic brain and OGD-insulted astrocytes. This regulatory effect of sevoflurane preconditioning on expression of NDRG2 might be associated with its function in modulating p53, an upstream mediator of NDRG2 in response to OGD treatment.[17] Accordingly, altered astrocyte-specific expression of NDRG2 might be as a part of changes in p53 proapoptotic signaling under sevoflurane preconditioning, which contributes to the neuroprotection by sevoflurane preconditioning.

The role of NDRG2 in cytotoxicity and cell death has been reported in a series studies. On the one hand, NDRG2 is relevant to cell survival by involvement in the Akt-mediated protection against cellular injury.[45,46] On the other, NDRG2 has been shown to promote the apoptotic cell death in some cells.[47,48] Hence, the distinct effects of NDRG2 on cell injury may be related to the different cell types and stimulations. Our recent study demonstrated that down-regulation of NDRG2 reduced the OGD-induced apoptosis in astrocytes originated from C6 glioma cells, indicating the potential proapoptotic role of NDRG2 in astrocytes.[17] In the current study, the NDRG2 knockdown by siRNA attenuated cellular apoptosis in astrocytes exposed to OGD, suggesting NDRG2-activated apoptosis signaling in astrocytes after OGD insult. Moreover, we observed that overexpression of NDRG2 partly reversed the protective effects of sevoflurane preconditioning. Accordingly, the suppression of NDRG2 up-regulation by sevoflurane preconditioning made it plausible to speculate that sevoflurane preconditioning induces the rapid tolerance to ischemic/hypoxia injury *via* inhibiting the up-regulation and nuclear translocation of NDRG2, suggesting that the interference of NDRG2 may be responsible for the neuroprotection of sevoflurane preconditioning.

There are some limitations in this study. First of all, we selected only one time point (24 h after reperfusion) to observe the role of NDRG2 in sevoflurane preconditioning–induced neuroprotection. In the matter of fact, this time point represents the neuroprotective effects by sevoflurane preconditioning in acute phase after reperfusion, but the involvement of NDRG2 in regulation of sevoflurane preconditioning in late phase after reperfusion (>72 h after reperfusion) is still unknown. Therefore, the participant of NDRG2 in the preconditioning effect of sevoflurane in the late phase after reperfusion deserves further study. Second, the mechanism underlying astrocyte-specific neuroprotection induced by sevoflurane in intact animals would be more complicated than what has been observed in astrocytes. Other than regulation of astrocytes, involvement of NDRG2 in astrocyte–neuron interaction might be a more important mechanism of sevoflurane preconditioning–induced neuroprotection. However, it is hard to achieve the efficient modulation of NDRG2 in intact animals by using vectors *in vivo*. Hence, further study is needed to investigate the role of NDRG2 in sevoflurane preconditioning by using NDRG2-specific knock-out mice or NDRG2-transgenic mice.

From bench to bedside, previous and our current experimental studies reveal that sevoflurane preconditioning should be a potential therapeutic strategy for patients undergoing surgery. According to basic research, sevoflurane preconditioning is promising to protect organs susceptible to

ischemia–reperfusion injury, such as heart, liver, and brain. Thus, a series of clinical trials have been developed to confirm its effectiveness in patients. In coronary bypass graft surgery, preconditioning with sevoflurane could decrease biochemical markers for myocardial dysfunction[49,50] and improve cardiovascular outcome.[51] However, this myocardial protective effect by sevoflurane preconditioning is still controversial in patients undergoing noncardiac surgery.[52] In addition, sevoflurane preconditioning significantly limited the increase of alanine transaminase and aspartate aminotransferase after liver surgery, demonstrating its potential role in protection of liver.[53] Although there is no report about neuroprotection of sevoflurane preconditioning in patients, ongoing trials that focus on this topic are carrying out in some institutes (NCT01204268; Xijing Hospital, Xi'an, China). Accordingly, evidence for relation between sevoflurane preconditioning and perioperative neuroprotection may be available in near future.

In conclusion, the current study demonstrates that sevoflurane preconditioning inhibits the up-regulation and nuclear translocation of NDRG2 in astrocytes, which provides a supportive evidence that sevoflurane preconditioning probably protects against cerebral ischemic damage *via* antiapoptosis signaling pathway. This may represent an important target in regulating function of astrocytes and NDRG2 for cell survival and proliferation after ischemic insult. Our data support the idea that neurons are not the only target for neuroprotection induced by sevoflurane preconditioning. Further elucidation of the relation between support cells, such as astrocytes, might provide a novel target for preventive and therapeutic strategies against cerebral ischemia–reperfusion injury.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Qiang Wang: Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, 127 Changle West Road, Xi'an 710032, Shaanxi Province, China. wangqiang@fmmu.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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