Astrocytic N-Myc Downstream-regulated Gene–2 Is Involved in Nuclear Transcription Factor κB–mediated Inflammation Induced by Global Cerebral Ischemia

You-liang Deng, M.D., Yu-long Ma, Ph.D., Zeng-li Zhang, M.D., Li-xia Zhang, M.D., Hang Guo, Ph.D., Pei Qin, M.D., Yu-shu Hou, M.D., Zi-jun Gao, M.D., Wu-gang Hou, Ph.D.

ABSTRACT

Background: Inflammation is a key element in the pathophysiology of cerebral ischemia. This study investigated the role of N-Myc downstream-regulated gene–2 in nuclear transcription factor κB–mediated inflammation in ischemia models.

Methods: Mice (n = 6 to 12) with or without nuclear transcription factor κB inhibitor pyrrolidinedithiocarbamate pretreatment were subjected to global cerebral ischemia for 20 min. Pure astrocyte cultures or astrocyte-neuron cocultures (n = 6) with or without pyrrolidinedithiocarbamate pretreatment were exposed to oxygen-glucose deprivation for 4 h or 2 h. Astrocytic nuclear transcription factor κB and N-Myc downstream-regulated gene–2 expression, proinflammatory cytokine secretion, neuronal apoptosis and survival, and memory function were analyzed at different time points after reperfusion or reoxygenation. Proinflammatory cytokine secretion was also studied in lentivirus-transfected astrocyte lines after reoxygenation.

Results: Astrocytic nuclear transcription factor κB and N-Myc downstream-regulated gene–2 expression and proinflammatory cytokine secretion increased after reperfusion or reoxygenation. Pyrrolidinedithiocarbamate pretreatment significantly reduced N-Myc downstream-regulated gene–2 expression and proinflammatory cytokine secretion *in vivo* and *in vitro*, reduced neuronal apoptosis induced by global cerebral ischemia/reperfusion (from 65 ± 4% to 47 ± 4%, $P = 0.0375$) and oxygen-glucose deprivation/reoxygenation (from 45.6 ± 0.2% to 22.0 ± 4.0%, $P < 0.001$), and improved memory function in comparison to vehicle-treated control animals subjected to global cerebral ischemia/reperfusion. N-Myc downstream-regulated gene–2 lentiviral knockdown reduced the oxygen-glucose deprivation-induced secretion of proinflammatory cytokines.

Conclusions: Astrocytic N-Myc downstream-regulated gene–2 is up-regulated after cerebral ischemia and is involved in nuclear transcription factor κB–mediated inflammation. Pyrrolidinedithiocarbamate alleviates ischemia-induced neuronal injury and hippocampal-dependent cognitive impairment by inhibiting increases in N-Myc downstream-regulated gene–2 expression and N-Myc downstream-regulated gene–2—mediated inflammation. **(Anesthesiology 2018; 128:574-86)**

GLOBAL cerebral ischemia (GCI), which selectively destroys CA1-region neurons in the hippocampus, occurs after cardiac arrest or systemic hypotension during surgery.[1] More than half of the patients who survive cardiac arrest suffer severe neurologic injury.[2,3] Emerging evidence suggests that neuroinflammation plays a critical role in the pathophysiology of postischemic brain injury and worsens neuronal injury.[4,5] The inhibition of inflammation has remarkable protective effects on the ischemic brain and has recently emerged as a promising therapeutic strategy.[6]

Astrocytes are critical for multiple functions of the central nervous system (CNS), including modulating synaptic plasticity; maintaining glucose, ion, and glutamate homeostasis; and maintaining the blood–brain barrier.[7–9] However, in response to cerebral ischemia, astrocytes become reactive, which results in the activation of immune mediators, the release of large quantities of proinflammatory cytokines, and neuronal dysfunction and death.[10,11] The heterodimeric transcription factor nuclear transcription factor κB (NF-κB) is a key regulator of inflammation in both the peripheral nervous system and the CNS.[12] Recent studies have reported that in the CNS, astrocytes are the primary cell type under NF-κB regulation, whereas neurons exhibit negligible NF-κB activity.[13,14] In addition, the selective inhibition

What We Already Know about This Topic

- Cerebral ischemia evokes a potent inflammatory response that results in astrocyte activation and nuclear transcription factor κB (NF-κB)–mediated elaboration of cytokines that can adversely impact neuronal survival.
- NF-κB activation leads to the elaboration of astrocyte-specific N-Myc downstream-regulated gene–2 (NDRG2); whether NDRG2 plays a role in neuronal injury is not clear.

What This Article Tells Us That Is New

- In *in vitro* cocultures of astrocytes and neurons, inhibition of nuclear transcription factor κB (NF-κB) or knockdown of N-Myc downstream-regulated gene–2 (NDRG2) in astrocytes reduced neuronal apoptosis. Similarly, inhibition of NF-κB *in vivo* reduced neuronal injury and better preserved learning and memory function in rodents.
- The results suggest that suppression of astrocyte-mediated inflammation can improve neuronal survival and that NDRG2 may serve as a pharmacologic target for the improvement of outcome after cerebral ischemia.

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of astroglial NF-κB signaling markedly alleviates ischemic injury in GAP-IκBα-dn transgenic mice.[15]

Our previous study revealed that in Leydig cells treated with the toxicant ethane dimethanesulfonate, NF-κB modulated the expression of N-Myc downstream-regulated gene–2 (NDRG2),[16] a newly identified differentiation- and stress-associated gene that is highly expressed in the heart, muscle, kidney, and brain.[17] Interestingly, our previous study and another study have found that in the mouse brain, NDRG2 is specifically expressed in astrocytes but not in neurons.[18,19] Additionally, we observed a significant upregulation of NDRG2 expression in the ischemic penumbra after cerebral ischemia induced by middle cerebral artery occlusion.[20] However, the specific role of NDRG2 in cerebral ischemic injury, especially in GCI, remains elusive. In addition, there are no reports on whether NDRG2 is involved in astrocytic NF-κB–mediated inflammation after cerebral ischemia. In this study, we investigated the role of NDRG2 in global cerebral ischemia–induced inflammatory and neuronal injury, which may reveal a new molecular target for cerebral ischemia therapy.

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 were performed in accordance with university guidelines for animal experimentation. Adult male C57BL/6 mice age 8 to 12 weeks and weighing 20 to 23 g, 1-day-old C57BL/6 pups, and pregnant C57BL/6 mice were provided by the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China).

Cell Culture

Primary Astrocyte Culture. Briefly, meninges-free hippocampal tissue from 1-day-old C57BL/6 mice was collected, minced, and dissociated with 0.025% trypsin to form a cell suspension, which was then added to Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum. The cells were cultured in 25 μg/ml poly-L-lysine–coated (Sigma-Aldrich, USA) T75 flasks and maintained at 37°C in a mixture of 95% atmospheric air and 5% CO_2. Half of the medium was replaced every 3 days. When the cells reached confluence after 3 days, the flasks were shaken at 190 rpm for 19 h to remove microglia and oligodendrocytes. After shaking, immunofluorescence staining for glial fibrillary acidic protein (GFAP) determined that more than 95% of the cultured cells were astrocytes, as shown in figure 1, Supplemental Digital Content, http://links.lww.com/ALN/B598. After purification, the cells were subcultured into different plates for various experiments.

Indirect Neuron–Astrocyte Coculture. Brains were isolated from 14- to 16-day-old embryos from pregnant C57 mice and used to culture primary hippocampal neurons using the same procedure described for primary astrocyte cultures except that neurobasal medium containing 1% glutamate and 2% B27 was used to culture the primary neurons. The neurons were cultured in 6-well plates. On the first day of culture, 5 μM Ara-C was added to the neuronal cultures to reduce glial cell contamination. Then, Transwells (3 μm, Corning, USA) containing cultured astrocytes were placed on top of the mature neuron cultures. The cocultures were then incubated at 37°C in a mixture of 95% atmospheric air and 5% CO_2 in neurobasal media containing 1% glutamate, 2% B27, and 10% fetal bovine serum.

Astrocyte Cell Culture. MA1800-57 cells were purchased from ScienCell (USA) and used as described in our previous study.[21]

Experimental Procedures

Experiment 1. The mice were randomly divided into the following three groups for *in vivo* studies: control, global cerebral ischemia/reperfusion (GCI/R), and pyrrolidinedithiocarbamate plus GCI/R. The mice in the control group underwent the same surgical procedure as the other mice but without bilateral common carotid artery ligation, whereas the mice in the GCI/R and pyrrolidinedithiocarbamate + GCI groups were subjected to GCI for 20 min followed by reperfusion for different durations. NF-κB, NDRG2, and proinflammatory cytokine (TNF-α, IL-6, and IL-1β) expression levels and locations were assessed at different time points (0 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 7 days) after reperfusion by Western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence. In addition, neuronal injury and survival were analyzed at 72 h or 7 days after reperfusion by hematoxylin and eosin (HE), terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL), and neuronal nuclei (NeuN) staining. Furthermore, hippocampal-dependent cognitive impairment was measured 7 days after reperfusion using the fear conditioning test.

Experiment 2. Pure primary astrocyte cultures and cocultures were assigned to the control, oxygen-glucose deprivation and reoxygenation plus vehicle (OGD/R + vehicle) or OGD/R + pyrrolidinedithiocarbamate group. NF-κB, NDRG2, and proinflammatory cytokine (TNF-α, IL-6, and IL-1β) expression levels and locations were analyzed at different time points (0 h, 2 h, 6 h, 12 h, and 24 h) after OGD/R by Western blotting and ELISA. In addition,

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neuronal apoptosis was assessed 24 h after reoxygenation *via* cleaved caspase-3 expression and flow-cytometric analyses.

The astrocyte cell lines were transfected with the lentivirus and divided into five groups: cells without lentiviral transfection (control group), cells transfected with a control shRNA or shRNA-NDRG2 (shRNA-control group and shRNA-NDRG2 group, respectively), and cells transfected with a control lentivirus (LEN)-beta-galactosidase (LEN-LacZ) or LEN-NDRG2 lentivirus (LV-Con group or LV-NDRG2 group, respectively). In addition, the levels of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in the culture medium were detected using ELISA 24 h after OGD/R.

GCI

GCI was induced in the C57BL/6 mice as described in our previous publication.[22] A laser Doppler flow meter (PeriFlux System 5000, Perimed, Sweden) was used to monitor cortical cerebral blood flow. Only the mice whose mean cortical cerebral blood flow decreased to 10% of the preischemic value and recovered to 80% of the baseline after reperfusion were used for data analysis (fig. 2, Supplemental Digital Content, http://links.lww.com/ALN/B598). The mice whose mean cortical cerebral blood flow did not decrease to 10% (n = 11) and those that died during ischemia (n = 9) were excluded. Homeostatic variables, including temperature, mean arterial blood pressure, and blood gas and glucose levels, were monitored in each animal before, during, and after ischemia (table 1, Supplemental Digital Content, http://links.lww.com/ALN/B598).

OGD

OGD/R was performed as described in our previous publication.[22] In brief, the medium was replaced by glucose-free Dulbecco's modified Eagle's medium, and the cells were placed in an anaerobic chamber, which was flushed for 15 min with 95% N_2 and 5% CO_2 and then sealed at 37°C for 4 h for astrocytes and 2 h for cocultures. After the 4-h or 2-h challenge, the cells were removed from the anaerobic chamber, and the medium was replaced with normal culture medium. Then, the cells were maintained in a cell incubator for different lengths of time to induce reoxygenation damage.

Drug Treatment

Pyrrolidinedithiocarbamate treatment was performed as described in previous studies.[23,24] For *in vivo* studies, pyrrolidinedithiocarbamate was dissolved in saline, and 200 mg/kg was administered by intraperitoneal injection 20 min before GCI. For *in vitro* studies, pyrrolidinedithiocarbamate was dissolved in dimethylsulfoxide and further diluted to a concentration of 50 μM, 100 μM, or 200 μM using Dulbecco's modifed Eagle's medium. Pyrrolidinedithiocarbamate was added 1 h before the onset of OGD exposure, and the final dimethylsulfoxide concentration was 0.003% (v/v).

Fear Conditioning Test

The fear conditioning test, which is mainly a learning test, was conducted over the course of 2 days using training and context protocols. Each mouse was individually placed in a control chamber to acclimate for 880 s on the seventh day after reperfusion. Then, on the eighth day, for training, they were subjected to 5 unsignaled footshocks (1 s in duration, 0.8 mA) that were randomly given during the 880-s period and occurred during the last second of a 20-s sound stimulus. On the ninth day, hippocampal-dependent contextual fear memory was tested without auditory electrical stimuli for 880 s by exposing the animals to the same context; freezing time, defined as a lack of movement except for respiration, was determined by visually inspecting a video.

Western Blotting Analysis

Proteins from the cultured cells and the hippocampal CA1 region were harvested at different time points and homogenized in RIPA lysis buffer (Beyotime, China) with 1 mM phenylmethylsulfonyl fluoride. Then, the collected proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, United Kingdom). The following antibodies were used for immunoblotting: rabbit anti-NDRG2 (1:1,000), rabbit anti–NF-κB (1:500), rabbit anticleaved caspase-3 (1:1,000), rabbit anti–pro–caspase-3 (1:1,000) and mouse anti–β-actin (1:1,000). The membranes were then incubated with an horseradish peroxidase–conjugated secondary antibody for 2 h. Protein bands were visualized using a LI-COR Odyssey System (LI-COR Biotechnology, USA).

Cell Fraction Assay

Twenty-four hours after the pure cultures were exposed to OGD, nuclear extracts were prepared in accordance with the protocol of the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, USA). Briefly, 200 μl of ice-cold cytoplasmic extraction reagent I was added to the washed astrocytes and incubated on ice for 10 min; then, 11 μl of ice-cold cytoplasmic extraction reagent II was added and incubated on ice for 1 min. The tube was then centrifuged in a microcentrifuge for 5 min at maximum speed (16,000 × *g*). The supernatant fraction (cytoplasmic extract) was immediately transferred to a clean prechilled tube, and 100 μl of ice-cold nuclear extraction reagent was added to the insoluble fraction by vortexing for 15 s every 10 min for a total of 40 min. The tube was then centrifuged for 10 min in a microcentrifuge at maximum speed. The nuclear extract fraction was then moved to a clean prechilled tube. All extracts were analyzed by Western blotting.

ELISA

The tissue fluid of the hippocampal CA1 region and the cell culture medium were collected at different time points, and ELISA reagent was used to quantitatively determine

the concentrations of proinflammatory cytokines (TNF-α, IL-6, and IL-1β). Absorbance was measured at 450 nm using a microplate reader (Infinite M2000, TECAN, Switzerland).

Immunofluorescence Staining

Paraffin sections were deparaffinized in xylene and rehydrated. The sections were incubated with proteinase K (10 μg/ml) for 15 min at 37°C for antigen retrieval, and nonspecific antibody binding sites were blocked with 1% bovine serum albumin–phosphate buffer saline. The sections were incubated overnight at 4°C with rabbit anti-NDRG2 (1:100), mouse anti-NDRG2 (1:100), mouse anti-GFAP (1:500), rabbit anti–NF-κB (1:100), rabbit anti–TNF-α (1:100) or mouse anti-NeuN (MAB377, 1:100, Millipore, USA) antibodies in 1% bovine serum albumin–phosphate buffer saline. The sections were then washed in tris-buffered saline with Tween and incubated with an antimouse fluorescein isothiocyanate (FITC)–tagged secondary antibody (1:200) and an antirabbit CY3-tagged secondary antibody (1:200) for 2 h at room temperature. The nuclei were subsequently stained with 4′,6-diamidino-2-phenylindole (1 ng/μl), and the sections were mounted with 50% glycerol and examined under a fluorescence microscope.

HE and TUNEL Staining

The mice that were subjected to 20 min of GCI were euthanatized 3 days after reperfusion, and their brains were fixed *via* perfusion with a buffered 4% paraformaldehyde solution, paraffin-embedded, and sectioned at a thickness of 4 μm for HE and TUNEL staining. HE staining was performed as previously described[25] for morphologic observation. TUNEL staining was performed using an *In Situ* Cell Death Detection kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The sections were treated with 0.3% (v/v) H_2O_2 for 20 min and then incubated in a TUNEL reaction mixture for 1 h at 37°C followed by converter-peroxidase for 30 min at 37°C. After washing 3 times in PBS, the sections were developed with 3,3′-diaminobenzidine for 5 min at room temperature. The number of TUNEL-positive neurons in the CA1 region was counted in 3 different fields for each section in a blinded manner using a light microscope at 400× magnification.

Flow-Cytometric Analysis

After OGD/R, neuronal apoptosis was assayed by flow cytometry. Briefly, the cells were washed with 1× annexin V–FITC binding buffer before staining with annexin V–FITC and propidium iodide (PI) for 15 min at room temperature in the dark. The stained cells were immediately analyzed by flow cytometry. Apoptotic and necrotic cells were quantitated based on annexin V binding and PI uptake. The annexin V–FITC$^+$/PI$^-$ and annexin V–FITC$^+$/PI$^+$ cell populations were considered to represent early and late apoptotic cells, respectively. In addition, the apoptosis index was calculated as the sum of the early and late apoptotic cell populations.

NDRG2 Overexpression and Interference

NDRG2 overexpression and interference in MA1800 cells were achieved *via* lentiviral transfection and were performed as described in our previous publication.[21]

Statistical Analysis

The determination of the sample size was mainly based on our previous experience.[22,26] All values are expressed as the mean ± SD. All analyses were performed using the SPSS software (version 14.0, USA). Differences between two groups were analyzed using Student's *t* tests. Differences among various groups were detected by two-way analysis of variance followed by the Tukey *post hoc* test. Variables that were measured repeatedly were compared using two-way repeated-measures analysis of variance. Two-tailed *P* values less than 0.05 were considered statistically significant.

Results

Ischemia-reperfusion Induced Significant Increases in NF-κB and NDRG2 Expression Levels and a Severe Inflammatory Response

First, we verified that NF-κB and NDRG2 colocalized in hippocampal astrocytes both *in vivo* and *in vitro*. Double immunofluorescence staining results revealed that NF-κB and NDRG2 primarily colocalized with the astrocyte marker GFAP in the hippocampal CA1 region *in vivo* (fig. 1A), demonstrating that NF-κB and NDRG2 were primarily expressed in astrocytes in the hippocampus. Additionally, we found that NF-κB colocalized with NDRG2 in the hippocampal CA1 region under normal conditions (fig. 1B, control group), and their expression significantly increased after GCI/R (fig. 1B). We also verified that NF-κB and NDRG2 colocalized in primary cultured hippocampal astrocytes *in vitro* (fig. 3, Supplemental Digital Content, http://links.lww.com/ALN/B598).

Next, we studied the effects of GCI/R on the expression of NF-κB and NDRG2 and on the inflammatory response. Compared to the control group, in the mice that experienced ischemia, the protein expression levels of both NF-κB and NDRG2 were significantly increased immediately after reperfusion (0 h) and were maintained at high levels up to 72 h after reperfusion (fig. 1C). In addition, the increase in NDRG2 expression was consistent with but slightly less than the increase in NF-κB expression. Proinflammatory cytokine (TNF-α, IL-6, and IL-1β) levels in the hippocampal CA1 region after GCI/R were studied using ELISA. As shown in figure 1D(a), compared to those in the control group, the expression levels of TNF-α and IL-6 markedly increased from 0 h to 72 h after reperfusion, but the levels of IL-1β expression were not significantly different between the control group and the GCI/R groups. Importantly, using double immunofluorescence staining, we found that TNF-α

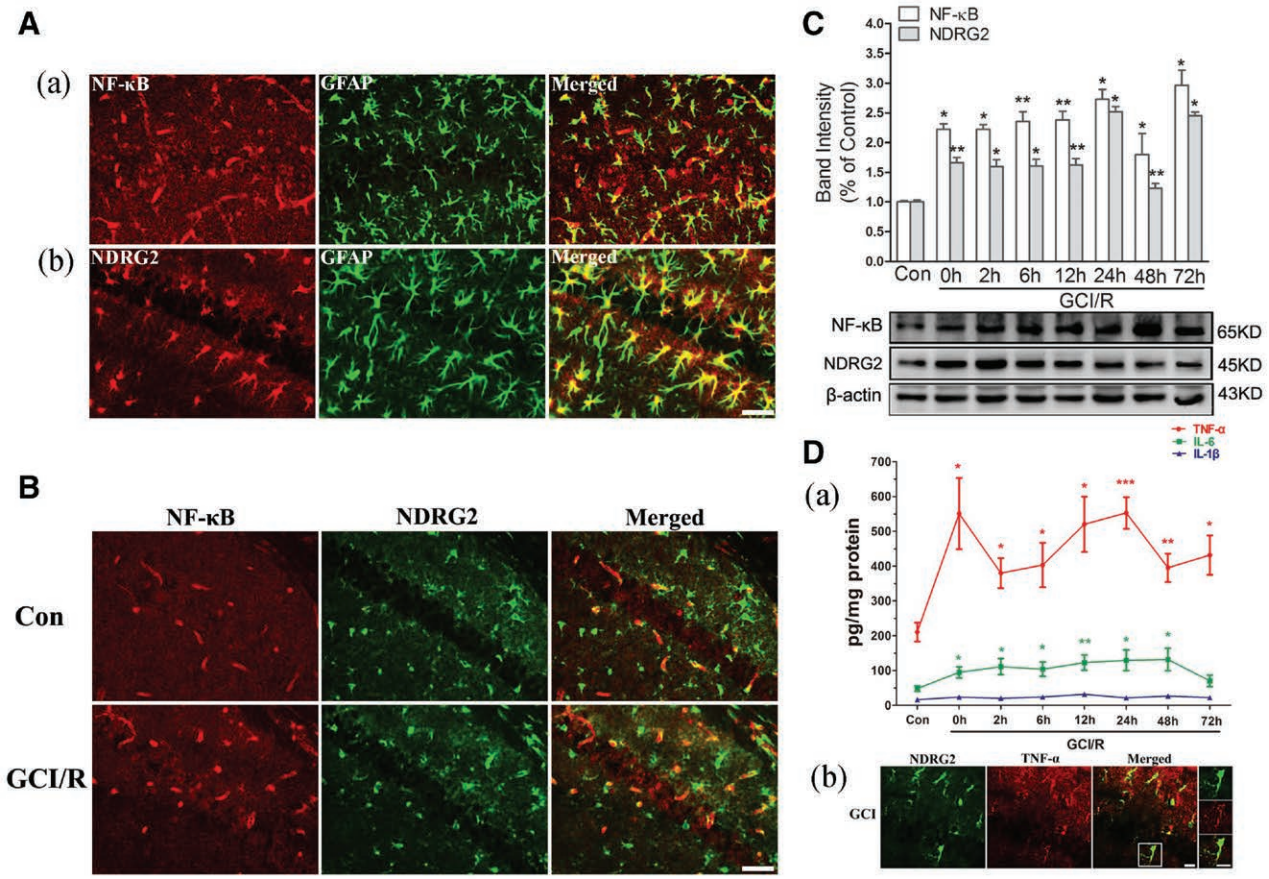

Fig. 1. Global cerebral ischemia/reperfusion (GCI/R) increased the expression of nuclear transcription factor κB (NF-κB), N-Myc downstream-regulated gene–2 (NDRG2), and proinflammatory cytokines in the hippocampal CA1 region. (*A*) Cellular localization of the NF-κB and NDRG2 proteins in the hippocampal CA1 region. NF-κB *(a)* and NDRG2 *(b)* largely overlapped with the astrocyte marker glial fibrillary acidic protein (GFAP) in the hippocampal CA1 region. *Scale bar*: 20 μm. (*B*) Localization and expression of NF-κB and NDRG2 in the hippocampal CA1 region after GCI/R. NDRG2 and NF-κB colocalized in the hippocampal CA1 region under normal conditions, and their expression increased after reperfusion (24 h). *Scale bar*: 20 μm. (*C*) The expression levels of the NF-κB and NDRG2 proteins were significantly up-regulated at different time points after GCI/R. The data are expressed as the mean ± SD (n = 6). **P* < 0.05 and ***P* < 0.01 *versus* the control (Con) group. (*D*) Expression of proinflammatory cytokines and colocalization of NDRG2 and TNF-α after GCI/R. *(a)* The expression levels of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) markedly increased after GCI/R. The data are expressed as the mean ± SD (n = 6). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 *versus* the Con group. *(b)* NDRG2 overlapped with TNF-α in the hippocampal CA1 region 24 h after reperfusion. *Scale bar*: 10 μm.

colocalized with NDRG2 in the hippocampal CA1 region after GCI/R (fig. 1D[b]).

Then, pure cultured hippocampal astrocytes were subjected to an OGD/R treatment to mimic *in vivo* GCI/R. Western blotting analysis revealed that the NF-κB and NDRG2 protein expression levels in astrocytes were markedly increased from 0 h to 24 h after OGD/R (fig. 2A). We also used ELISA to detect the levels of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) that were secreted by astrocytes into the culture medium at different time points after OGD/R. The results showed that the secretion of TNF-α and IL-6 increased immediately at 0 h and 2 h and reached a maximum 24 h after OGD/R (fig. 2B). However, the secretion of IL-1β was only significantly increased 24 h after OGD/R injury (fig. 2B; **P* = 0.0495 *vs*. the control group).

Inhibition of NF-κB Activity Significantly Inhibited the Increase in NDRG2 and Proinflammatory Cytokines Induced by GCI/R or OGD/R

We used pyrrolidinedithiocarbamate, a specific inhibitor of NF-κB activity, to explore the role of NDRG2 in NF-κB–mediated inflammation after ischemia. As shown in figure 3A, NDRG2 expression was markedly increased 1, 2, and 3 days after GCI/R, whereas treatment with pyrrolidinedithiocarbamate 20 min before GCI surgery dramatically reduced the increase in NDRG2 expression. In addition, pyrrolidinedithiocarbamate treatment significantly reduced the increases in the proinflammatory cytokines TNF-α and IL-6 in the hippocampal CA1 region after GCI/R injury (fig. 3, B and C).

In vitro experiments showed that NF-κB expression significantly increased both in the cytoplasm and in the nucleus after

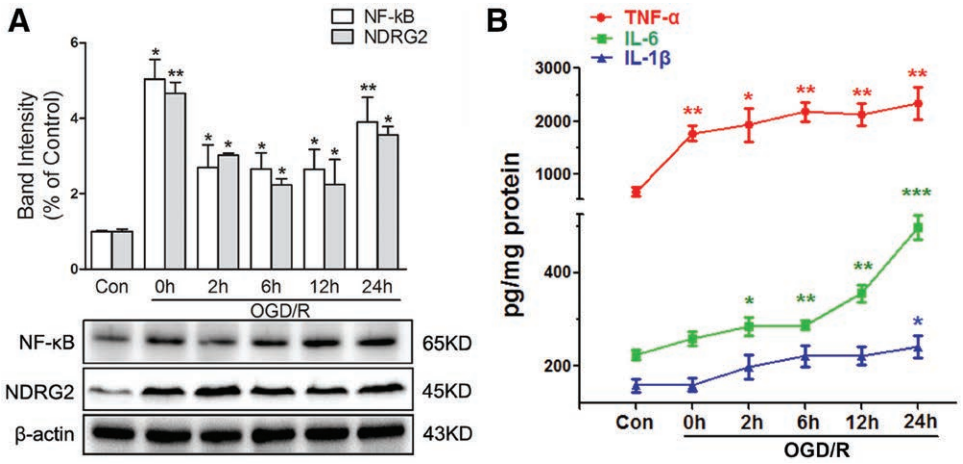

Fig. 2. Expression of N-Myc downstream-regulated gene–2 (NDRG2) and nuclear transcription factor κB (NF-κB) and secretion of proinflammatory cytokines in cultured primary astrocytes exposed to oxygen-glucose deprivation and reoxygenation (OGD/R). NDRG2 and NF-κB protein expression levels (*A*) and inflammatory cytokine secretion (*B*) were markedly increased after OGD/R. The data are expressed as the mean ± SD (n = 6). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 *versus* the control (Con) group.

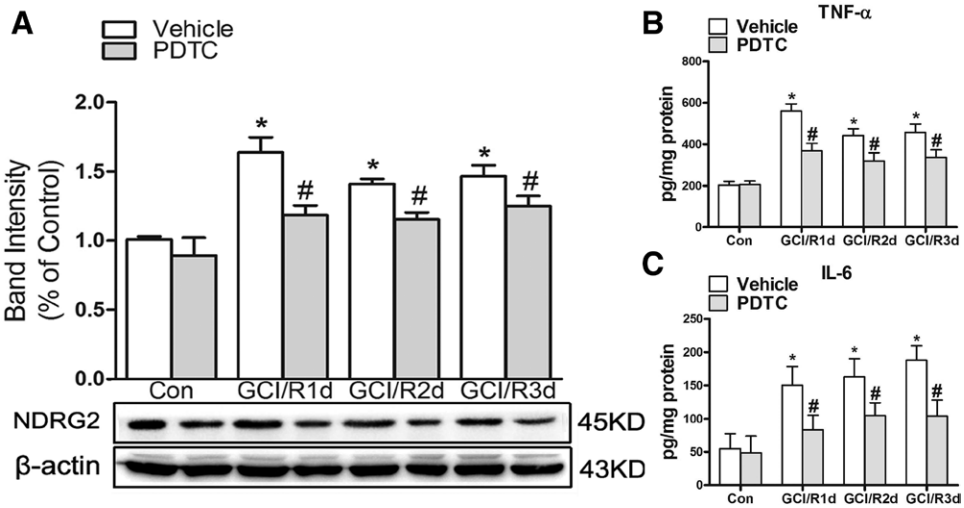

Fig. 3. Pyrrolidinedithiocarbamate (PDTC) pretreatment reduced the increase in N-Myc downstream-regulated gene–2 (NDRG2) and proinflammatory cytokine expression after global cerebral ischemia/reperfusion (GCI/R). PDTC pretreatment significantly reduced the GCI/R-induced increase in the expression levels of NDRG2 (*A*) and the proinflammatory cytokines TNF-α (*B*) and IL-6 (*C*). The data are expressed as the mean ± SD (n = 6). **P* < 0.05 *versus* the control (Con) group, and #*P* < 0.05 *versus* the GCI/R + vehicle group.

OGD/R injury and demonstrated the nuclear translocation of NF-κB after OGD/R injury (fig. 4A). However, pyrrolidinedithiocarbamate treatment reduced the increase in NF-κB expression in the nucleus without influencing its expression in the cytoplasm (fig. 4A). In addition, we analyzed the effects of different doses of pyrrolidinedithiocarbamate (50 μM, 100 μM, and 200 μM) on NDRG2 expression after OGD/R (fig. 4, Supplemental Digital Content, http://links.lww.com/ALN/B598) and found that treatment with 100 μM pyrrolidinedithiocarbamate significantly reduced the increase in NDRG2 expression induced by OGD/R (fig. 4B). Moreover, pyrrolidinedithiocarbamate treatment dramatically reduced the increase in the expression of the proinflammatory cytokines TNF-α and IL-6 after OGD/R injury (fig. 4, C and D).

Inactivation of NF-κB Alleviated the Neuronal Damage Induced by Ischemia-reperfusion

In *in vivo* experiments, GCI/R-injured neurons in the hippocampal CA1 region were detected using HE, TUNEL, and immunofluorescence staining. As revealed by HE staining 3 days after GCI surgery (fig. 5A), the neurons remained intact in the control group, and abundant nuclei centered in the cytoplasm were clearly stained. In contrast, neurons were severely damaged and lacked normal morphology after GCI/R, but pyrrolidinedithiocarbamate treatment reduced the neuronal damage. TUNEL staining 3 days after GCI surgery revealed that the percentage of TUNEL-positive cells in the CA1 region of the hippocampus was 65 ± 4% in the GCI/R group and that treatment with pyrrolidinedithiocarbamate significantly

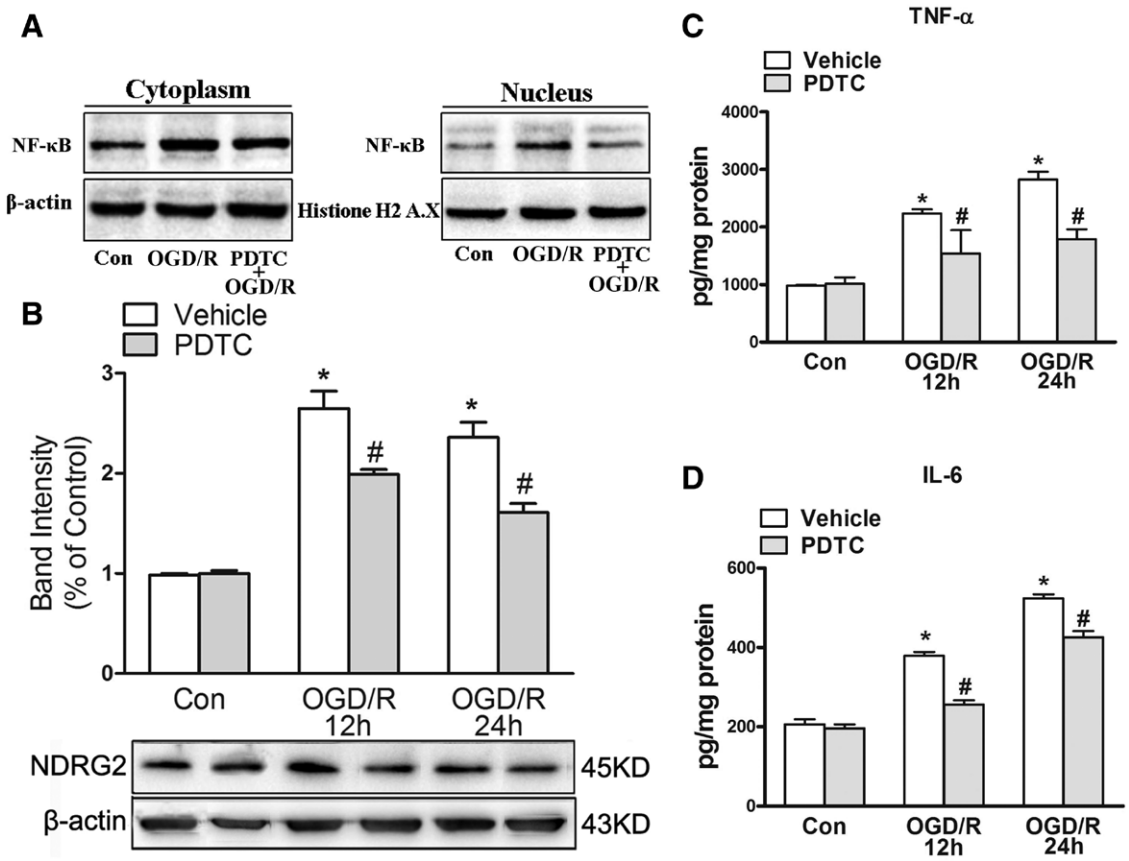

Fig. 4. Pyrrolidinedithiocarbamate (PDTC) inhibited nuclear transcription factor κB (NF-κB) nuclear translocation and reduced the increase in N-Myc downstream-regulated gene–2 (NDRG2) and proinflammatory cytokine expression induced by oxygen-glucose deprivation/reoxygenation (OGD/R). (*A*) Oxygen-glucose deprivation (OGD) induced the nuclear translocation of NF-κB, and PDTC pretreatment significantly inhibited this nuclear translocation. (*B*) PDTC markedly reduced the increase in NDRG2 expression induced by OGD/R. The data are expressed as the mean ± SD (n = 6). **P* < 0.05 *versus* the control (Con) group, and #*P* < 0.05 *versus* the OGD/R + vehicle group. (*C and D*) PDTC markedly reduced the OGD/R-induced increase in proinflammatory cytokine (TNF-α and IL-6) secretion by primary astrocytes. The data are expressed as the mean ± SD (n = 6). **P* < 0.05 *versus* the Con group, and #*P* < 0.05 *versus* the OGD/R + vehicle group.

reduced the percentage of TUNEL-positive cells to 46 ± 4% (fig. 5B). We also evaluated the survival of neurons *via* NeuN staining 7 days after GCI/R. As shown in figure 5B, the percentage of NeuN-positive cells in the hippocampal CA1 region was 44 ± 4% in the GCI/R group, and treatment with pyrrolidinedithiocarbamate significantly increased the percentage of NeuN-positive cells to 69 ± 4% (***P* = 0.0042 *vs.* the control group; ##*P* = 0.0058 *vs.* the GCI/R group).

Seven days after GCI surgery, we performed a fear conditioning test, which is a hippocampal-dependent cognitive test, to evaluate hippocampal neuronal injury. The movement track of the mice (fig. 5A, Supplemental Digital Content, http://links.lww.com/ALN/B598) and freezing time before the foot-shocks (fig. 5B, Supplemental Digital Content, http://links.lww.com/ALN/B598) showed that there were no differences among the control, control + pyrrolidinedithiocarbamate, GCI/R, and pyrrolidinedithiocarbamate + GCI/R groups. As shown in figure 5C, the freezing times of the mice in the control group and the control + pyrrolidinedithiocarbamate group were 39 ± 3% and 38 ± 7%, respectively. GCI/R significantly reduced the freezing time to 23 ± 2% that of the control, and pretreatment with pyrrolidinedithiocarbamate significantly increased the freezing time to 31 ± 2% that of the control.

In *in vitro* experiments, OGD/R was induced in astrocyte and neuron cocultures, and flow cytometry and Western blotting for cleaved caspase-3 were used to explore the effects of pyrrolidinedithiocarbamate on neuronal apoptosis. As shown in figure 6A, 24 h after reoxygenation, the apoptotic index of the neurons in the OGD group was 45.6 ± 0.2%. In addition, pretreating astrocytes with pyrrolidinedithiocarbamate (100 μM) dramatically attenuated OGD/R-induced neuronal apoptosis to 22.0 ± 4.0% (fig. 6A). As shown in figure 6B, 24 h after reoxygenation, cleaved caspase-3 expression in the neurons had significantly increased in the OGD group, and pretreating astrocytes with pyrrolidinedithiocarbamate markedly decreased cleaved caspase-3 expression in neurons.

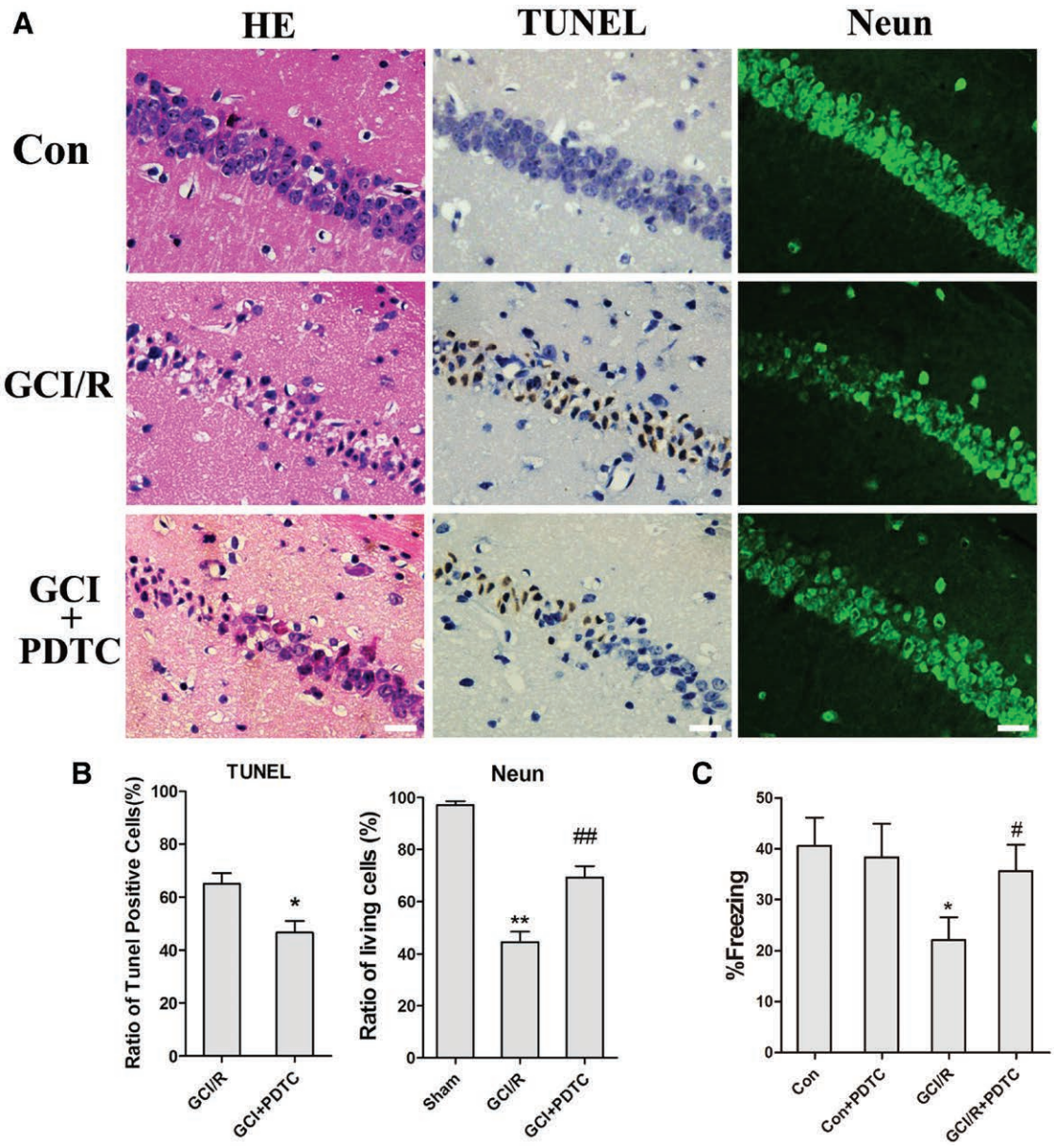

Fig. 5. Pyrrolidinedithiocarbamate (PDTC) pretreatment alleviated the neuronal injury and hippocampal-dependent cognitive impairment induced by global cerebral ischemia/reperfusion (GCI/R). (*A*) PDTC pretreatment significantly increased the number of intact neurons, as detected by hematoxylin and eosin (HE) staining 72 h after reperfusion; reduced neuronal apoptosis, as detected by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining 72 h after reperfusion; and increased neuronal survival, as detected by neuronal nuclei (Neun) staining 7 days after reperfusion. Scale bar: 20 μm. (*B*) The data are expressed as the mean ± SD (n = 8). **P* = 0.0375 *versus* the GCI/R group, ***P* = 0.0042 *versus* the control (Con) group, and ##*P* = 0.0058 *versus* the GCI/R group. (*C*) PDTC significantly improved the hippocampal-dependent cognitive impairment induced by GCI/R. The data are expressed as the mean ± SD (n = 12). **P* = 0.033 *versus* the Con group, and #*P* = 0.046 *versus* the GCI/R group.

Downregulation of NDRG2 Expression Alleviated the Inflammation Induced by OGD/R

To detect the direct relationship between NDRG2 and proinflammatory cytokine secretion after OGD/R, we used lentiviruses to transfect MA1800 astrocytes. First, we successfully established MA1800 cell lines in which NDRG2 expression was stably up- or down-regulated, as verified by Western blotting (fig. 7A). Then, we detected proinflammatory cytokine secretion from the MA1800 cells after OGD and 24 h of reoxygenation. As shown in figure 7B, in the shRNA-control group, the levels of TNF-α, IL-6, and IL-1β were 2,204 ± 472, 491 ± 76 and 222 ± 42 pg/mg protein, respectively. In addition, the downregulation of NDRG2 expression by shRNA-NDRG2 decreased the levels of TNF-α, IL-6, and IL-1β to 1,282 ± 322 (**P* = 0.0491), 344 ± 50 (**P* = 0.0495), and 144 ± 21 pg/mg

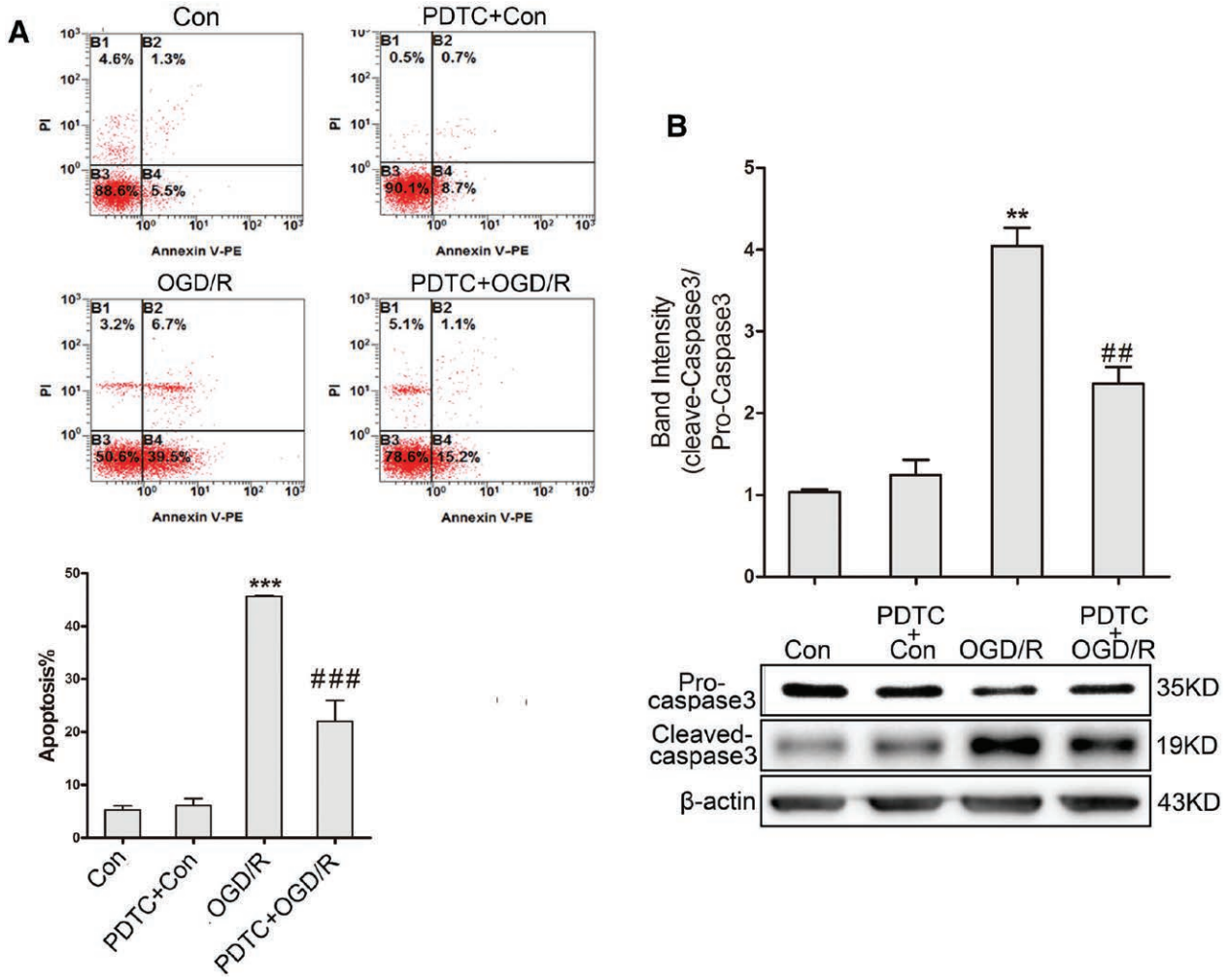

Fig. 6. Pyrrolidinedithiocarbamate (PDTC) pretreatment alleviated oxygen-glucose deprivation/reoxygenation (OGD/R)-induced neuronal apoptosis. (*A*) PDTC pretreatment significantly reduced neuronal apoptosis in astrocyte and neuron cocultures exposed to oxygen-glucose deprivation (OGD) for 2 h followed by reoxygenation for 24 h. The data are expressed as the mean ± SD (n = 6). ***P < 0.001 *versus* the control (Con) group, and ###P < 0.001 *versus* the OGD + vehicle group. (*B*) PDTC pretreatment significantly reduced the expression of the apoptotic protein cleaved caspase-3 in neurons after astrocyte and neuron cocultures were exposed to OGD for 2 h followed by reoxygenation for 24 h. The data are expressed as the mean ± SD (n = 6). **P = 0.0054 *versus* the Con group, and ##P = 0.0037 *versus* the OGD + vehicle group. PE = phycoerythrin; PI = propidium iodide.

protein (*P = 0.0448), respectively; these levels were significantly lower than those in the shRNA-control group. In the LV-control group, the levels of TNF-α, IL-6, and IL-1β were 2,471 ± 532, 521 ± 66, and 247 ± 50 pg/mg protein, respectively. NDRG2 overexpression *via* LV-NDRG2 markedly increased the levels of TNF-α, IL-6, and IL-1β to 3,598 ± 419 (#P = 0.0449), 712 ± 89 (#P = 0.0401), and 262 ± 48 pg/mg protein (#P = 0.7327), respectively; these levels were higher than those in the LV-control group.

Discussion

Neurons have been the focus of numerous therapeutic strategies for cerebral ischemia, all of which have failed to yield favorable patient outcomes. However, in the last decade, a growing body of evidence has indicated that astrocytes may be a viable target for cerebral ischemia therapy during both the acute phase of ischemia and the recovery period.[27] Under transient focal cerebral ischemia, astrocytes can distribute functional mitochondria to adjacent neurons, which promotes neuronal viability and recovery.[28] In addition, the upregulation of GLT-1 expression in CA1 astrocytes—but not DG or CA3 astrocytes—significantly reduced CA1 neuronal death after transient forebrain ischemia.[1] Furthermore, astrocytes promote axon regeneration by releasing multiple axon growth–supporting molecules after severe spinal cord injury.[29] However, within minutes after ischemic injury, injured neurons and glial cells in the core and ischemic penumbra produce proinflammatory mediators, cytokines, and reactive oxygen species, which activate astrocytes.[30] Activated astrocytes can produce high levels of proinflammatory cytokines, including IL-6, TNF-α, and IL-1β,[30] which hinder

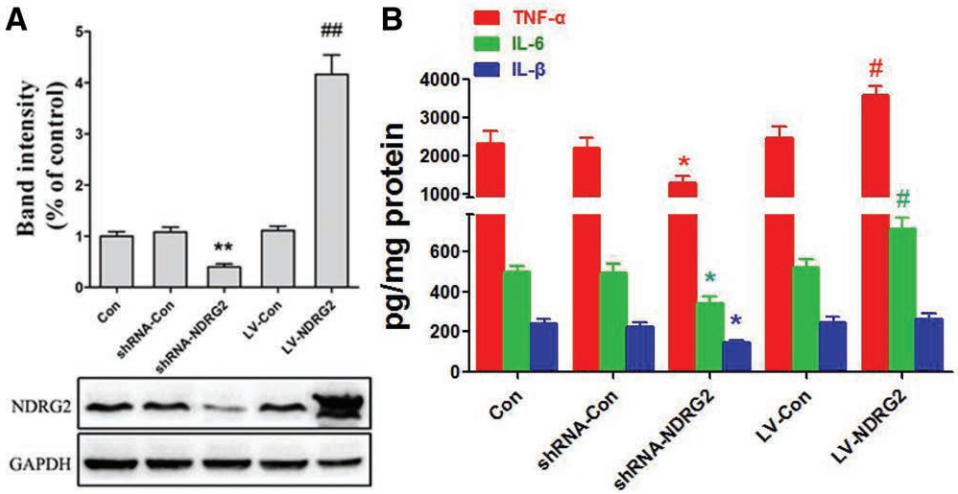

Fig. 7. Lentiviral knockdown of N-Myc downstream-regulated gene–2 (NDRG2) expression reduced the increase in proinflammatory cytokine secretion induced by oxygen-glucose deprivation/reoxygenation (OGD/R). (*A*) Verification of the lentiviral transfection of the astrocyte cell line (MA1800 cells) by Western blotting. The data are expressed as the mean ± SD (n = 6). $^{**}P < 0.01$ *versus* the shRNA-Con group, and $\#\#P < 0.01$ *versus* the LV-Con group. (*B*) Silencing NDRG2 significantly reduced the secretion of TNF-α, IL-6, and IL-1β, whereas overexpressing NDRG2 significantly increased the secretion of TNF-α and IL-6. The data are expressed as the mean ± SD (n = 6). $^{*}P < 0.05$ *versus* the shRNA-Con group, and $\#P < 0.05$ *versus* the LV-Con group. Con = control; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; LV = lentivirus; sh = short hairpin.

ischemic recovery by directly inducing neuronal apoptosis[31] and/or increasing toxic nitric oxide levels[1] and inhibiting neurogenesis.[32] In addition, inhibiting astrocyte-induced inflammation could alleviate ischemic injury,[15] which may provide a new therapeutic strategy for cerebral ischemia.

As mentioned, NF-κB is a key regulator of inflammation in the CNS, and astrocytes are the primary cell type under NF-κB regulation.[12–14] NF-κB is a transcription factor that is normally bound by its inhibitory protein, IκBα, and is sequestered in the cytoplasm. Ischemia triggers the degradation of IκBα by the ubiquitin proteasome system, resulting in the translocation of NF-κB to the nucleus, where it regulates gene transcription.[33] In this study, we found that after GCI/R or OGD/R, astrocytic NF-κB expression was markedly increased, and proinflammatory cytokine expression also increased, which indicates that astrocytic NF-κB is involved in ischemia-induced inflammatory injury. However, the question of whether inhibiting astrocytic NF-κB activity can alleviate ischemic injury remains controversial. In a previous study, transgenic mice were generated that expressed the IκBα super-repressor under the transcriptional control of the GFAP promoter; this study reported that the selective inhibition of astrocytic NF-κB did not reduce infarct size or cell death 48 h after permanent middle cerebral artery occlusion.[34] However, a recent study using GAP-IκBα-dn transgenic mice found that the selective inhibition of astroglial NF-κB significantly reduced the retinal ischemia–induced loss of retinal neurons.[15] Pyrrolidinedithiocarbamate is an inhibitor of NF-κB that stops the degradation of IκBα and the translocation of NF-κB into the nucleus,[35] and pyrrolidinedithiocarbamate treatment can reduce infarct volumes and improve neurologic behavior that has been impaired by focal cerebral ischemic injury.[36] However, the effects of pyrrolidinedithiocarbamate on GCI have rarely been reported. GCI selectively destroys the hippocampal CA1 region and causes severe hippocampal-dependent cognitive impairment,[37] and the current study revealed that pyrrolidinedithiocarbamate significantly reduced the effects of GCI/R, including the increased levels of proinflammatory cytokines (TNF-α and IL-6) and neuronal injury, as well as hippocampal-dependent cognitive impairment. More importantly, *in vitro* experiments showed that pyrrolidinedithiocarbamate treatment markedly reduced the translocation of astrocytic NF-κB into the nucleus and dramatically decreased the levels of proinflammatory cytokines and neuronal apoptosis induced by OGD/R, thus demonstrating the neuroprotective effects of the inhibition of astrocytic NF-κB signaling. However, the detailed molecular mechanisms have not been well defined.

NDRG2 is specifically expressed in astrocytes in the brain[17,18] and is involved in astrocyte activation and proliferation.[38] Additionally, NDRG2 is associated with the pathogenesis of neurodegenerative disorders, including Alzheimer disease,[39] depression,[40] and frontotemporal lobar degeneration.[41] We recently reported the spatial-temporal expression of NDRG2 in rat and mouse brains after middle cerebral artery occlusion injury;[20,21] our report indicated that astrocytic NDRG2 played an important role in cerebral ischemia. However, the specific role of NDRG2 in GCI injury and the underlying mechanisms have not been reported. In this study, we demonstrated that astrocytic NDRG2 expression markedly increased after GCI surgery or OGD injury, which was in accordance with the findings of our previous studies.[20,21] Interestingly, we found that the increase in NDRG2

expression was consistent with the increase in NF-κB and proinflammatory cytokine expression both *in vivo* and *in vitro* and that NDRG2 clearly colocalized with TNF-α in the hippocampal CA1 region, which indicated that NDRG2 may be involved in NF-κB–mediated inflammation after cerebral ischemia. In addition, our previous study revealed that NF-κB increased the expression of NDRG2 by binding to its promoter region in Leydig cells.[16] In this study, we found that pyrrolidinedithiocarbamate treatment significantly reduced the increase in NDRG2 expression induced by GCI/R or OGD/R. Based on these results, we hypothesize that ischemic stimuli activate NF-κB signaling, inducing its translocation from the cytoplasm to the nucleus, where it binds to the *ndrg2* gene promoter region to promote NDRG2 expression.

Although pyrrolidinedithiocarbamate reduced the expression of NDRG2 and proinflammatory cytokines, this finding is not definitive proof that there is direct regulation between NDRG2 and proinflammatory cytokine expression. Using lentiviruses, we found that silencing NDRG2 expression markedly reduced the secretion of proinflammatory cytokines in the medium after OGD/R, whereas the overexpression of NDRG2 increased the secretion of TNF-α and IL-6, which indicated that NDRG2 could directly regulate proinflammatory cytokine expression after cerebral ischemia. A recent study on a mouse model of cortical stab injury reported that deleting NDRG2 attenuated reactive astrogliosis and inflammatory responses through the regulation of IL-6/STAT3 signaling.[42] In addition, our previous study revealed that NDRG2 translocated to the nucleus after cerebral ischemia, which suggests that NDRG2 may be a novel transcription factor.[21] Therefore, we hypothesized that these proinflammatory cytokines may be NDRG2 target genes; this hypothesis warrants further exploration.

In addition, we previously found that electroacupuncture or sevoflurane pretreatment alleviated ischemic injury by inhibiting the increase in NDRG2 expression and NDRG2 nuclear translocation.[26,43] Therefore, we proposed that astrocytic NDRG2 may be a novel molecular target in cerebral ischemia therapy;[21] however, the additional roles of astrocytic NDRG2 in cerebral ischemia remain elusive. In this study, we found that NDRG2 was involved in NF-κB–mediated inflammation induced by cerebral ischemia, which provides a novel direction for the study of NDRG2 in cerebral ischemia.

There are some limitations to the current study. A substantial and growing body of evidence suggests that inflammatory microglia also contribute to the pathophysiology of neuronal death after GCI/R,[44,45] but we were not able to determine whether the proinflammatory cytokines were secreted by astrocytes or microglia in our *in vivo* experiments. However, we analyzed the colocalization of astrocytic NDRG2 and proinflammatory cytokines after GCI/R, and *in vitro* experiments further demonstrated the direct relationship between NDRG2 and proinflammatory cytokine expression in astrocytes. Therefore, our results suggest that astrocytic NDRG2 is involved in the inflammatory responses induced by cerebral ischemia, and its underlying molecular mechanisms will be the focus of our future studies.

In conclusion, the current study provides the first evidence that astrocytic NDRG2 is significantly up-regulated after GCI/R and is involved in NF-κB–mediated inflammation. Furthermore, we found that the NF-κB activation inhibitor pyrrolidinedithiocarbamate could alleviate ischemia-induced neuronal injury by inhibiting the increase in NDRG2 expression and NDRG2-mediated inflammation. These results reveal a new mechanism for this disorder and provide evidence that may be used to develop novel stroke therapies that target astrocytic NDRG2.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Hou: Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China. gangwuhou@163.com. 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.

References

1. Ouyang YB, Voloboueva LA, Xu LJ, Giffard RG: Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. J Neurosci 2007; 27:4253–60
2. Sasson C, Rogers MA, Dahl J, Kellermann AL: Predictors of survival from out-of-hospital cardiac arrest: A systematic review and meta-analysis. Circ Cardiovasc Qual Outcomes 2010; 3:63–81
3. Geocadin RG, Koenig MA, Jia X, Stevens RD, Peberdy MA: Management of brain injury after resuscitation from cardiac arrest. Neurol Clin 2008; 26:487–506, ix
4. Chamorro Á, Dirnagl U, Urra X, Planas AM: Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol 2016; 15:869–81
5. Gan Y, Liu Q, Wu W, Yin JX, Bai XF, Shen R, Wang Y, Chen J, La Cava A, Poursine-Laurent J, Yokoyama W, Shi FD: Ischemic neurons recruit natural killer cells that accelerate brain infarction. Proc Natl Acad Sci U S A 2014; 111:2704–9
6. Koh HS, Chang CY, Jeon SB, Yoon HJ, Ahn YH, Kim HS, Kim IH, Jeon SH, Johnson RS, Park EJ: The HIF-1/glial TIM-3

axis controls inflammation-associated brain damage under hypoxia. Nat Commun 2015; 6:6340
7. Coulter DA, Eid T: Astrocytic regulation of glutamate homeostasis in epilepsy. Glia 2012; 60:1215–26
8. Barreto GE, Gonzalez J, Torres Y, Morales L: Astrocytic-neuronal crosstalk: Implications for neuroprotection from brain injury. Neurosci Res 2011; 71:107–13
9. Seifert G, Steinhäuser C: Neuron-astrocyte signaling and epilepsy. Exp Neurol 2013; 244:4–10
10. Kyritsis N, Kizil C, Brand M: Neuroinflammation and central nervous system regeneration in vertebrates. Trends Cell Biol 2014; 24:128–35
11. Jian Z, Ding S, Deng H, Wang J, Yi W, Wang L, Zhu S, Gu L, Xiong X: Probenecid protects against oxygen-glucose deprivation injury in primary astrocytes by regulating inflammasome activity. Brain Res 2016; 1643:123–9
12. Baltimore D: NF-κB is 25. Nat Immunol 2011; 12:683–5
13. Lian H, Shim DJ, Gaddam SS, Rodriguez-Rivera J, Bitner BR, Pautler RG, Robertson CS, Zheng H: IκBα deficiency in brain leads to elevated basal neuroinflammation and attenuated response following traumatic brain injury: Implications for functional recovery. Mol Neurodegener 2012; 7:47
14. Mao XR, Moerman-Herzog AM, Chen Y, Barger SW: Unique aspects of transcriptional regulation in neurons–Nuances in NFkappaB and Sp1-related factors. J Neuroinflammation 2009; 6:16
15. Dvoriantchikova G, Barakat D, Brambilla R, Agudelo C, Hernandez E, Bethea JR, Shestopalov VI, Ivanov D: Inactivation of astroglial NF-kappa B promotes survival of retinal neurons following ischemic injury. Eur J Neurosci 2009; 30:175–85
16. Li T, Hu J, He GH, Li Y, Zhu CC, Hou WG, Zhang S, Li W, Zhang JS, Wang Z, Liu XP, Yao LB, Zhang YQ: Up-regulation of NDRG2 through nuclear factor-kappa B is required for Leydig cell apoptosis in both human and murine infertile testes. Biochim Biophys Acta 2012; 1822:301–13
17. Qu X, Zhai Y, Wei H, Zhang C, Xing G, Yu Y, He F: Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family. Mol Cell Biochem 2002; 229:35–44
18. Ma YL, Qin P, Feng DY, Li Y, Zhang LX, Liu ZY, Yin AQ, Tang WH, Dong HL, Meng LZ, Hou WG, Xiong LZ: Estrogen regulates the expression of Ndrg2 in astrocytes. Brain Res 2014; 1569:1–8
19. Okuda T, Kokame K, Miyata T: Differential expression patterns of NDRG family proteins in the central nervous system. J Histochem Cytochem 2008; 56:175–82
20. Li Y, Shen L, Cai L, Wang Q, Hou W, Wang F, Zeng Y, Zhao G, Yao L, Xiong L: Spatial-temporal expression of NDRG2 in rat brain after focal cerebral ischemia and reperfusion. Brain Res 2011; 1382:252–8
21. Ma YL, Zhang LX, Liu GL, Fan Y, Peng Y, Hou WG: N-Myc downstream-regulated gene 2 (Ndrg2) is involved in ischemia-hypoxia-induced astrocyte apoptosis: A novel target for stroke therapy. Mol Neurobiol 2017; 54:3286–99
22. Ma Y, Guo H, Zhang L, Tao L, Yin A, Liu Z, Li Y, Dong H, Xiong L, Hou W: Estrogen replacement therapy-induced neuroprotection against brain ischemia-reperfusion injury involves the activation of astrocytes via estrogen receptor β. Sci Rep 2016; 6:21467
23. Koyama Y, Tsujikawa K, Matsuda T, Baba A: Endothelin-1 stimulates glial cell line-derived neurotrophic factor expression in cultured rat astrocytes. Biochem Biophys Res Commun 2003; 303:1101–5
24. Nurmi A, Lindsberg PJ, Koistinaho M, Zhang W, Juettler E, Karjalainen-Lindsberg ML, Weih F, Frank N, Schwaninger M, Koistinaho J: Nuclear factor-kappaB contributes to infarction after permanent focal ischemia. Stroke 2004; 35:987–91
25. Sun BZ, Chen L, Wu Q, Wang HL, Wei XB, Xiang YX, Zhang XM: Suppression of inflammatory response by flurbiprofen following focal cerebral ischemia involves the NF-κB signaling pathway. Int J Clin Exp Med 2014; 7:3087–95
26. Li X, Luo P, Wang F, Yang Q, Li Y, Zhao M, Wang S, Wang Q, Xiong L: Inhibition of N-myc downstream-regulated gene-2 is involved in an astrocyte-specific neuroprotection induced by sevoflurane preconditioning. Anesthesiology 2014; 121:549–62
27. Liu Z, Chopp M: Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. Prog Neurobiol 2016; 144:103–20
28. Hayakawa K, Esposito E, Wang X, Terasaki Y, Liu Y, Xing C, Ji X, Lo EH: Transfer of mitochondria from astrocytes to neurons after stroke. Nature 2016; 535:551–5
29. Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, Coppola G, Khakh BS, Deming TJ, Sofroniew MV: Astrocyte scar formation aids central nervous system axon regeneration. Nature 2016; 532:195–200
30. Tuttolomondo A, Di Raimondo D, di Sciacca R, Pinto A, Licata G: Inflammatory cytokines in acute ischemic stroke. Curr Pharm Des 2008; 14:3574–89
31. Zhao JJ, Hu JX, Lu DX, Ji CX, Qi Y, Liu XY, Sun FY, Huang F, Xu P, Chen XH: Soluble cpg15 from astrocytes ameliorates neurite outgrowth recovery of hippocampal neurons after mouse cerebral ischemia. J Neurosci 2017; 37:1628–47
32. Monje ML, Toda H, Palmer TD: Inflammatory blockade restores adult hippocampal neurogenesis. Science 2003; 302:1760–5
33. Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A, Ben-Neriah Y: Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 1995; 92:10599–603
34. Zhang W, Potrovita I, Tarabin V, Herrmann O, Beer V, Weih F, Schneider A, Schwaninger M: Neuronal activation of NF-kappaB contributes to cell death in cerebral ischemia. J Cereb Blood Flow Metab 2005; 25:30–40
35. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K: Evidence that reactive oxygen species do not mediate NF-kappaB activation. EMBO J 2003; 22:3356–66
36. Pfeilschifter W, Czech B, Hoffmann BP, Sujak M, Kahles T, Steinmetz H, Neumann-Haefelin T, Pfeilschifter J: Pyrrolidine dithiocarbamate activates p38 MAPK and protects brain endothelial cells from apoptosis: A mechanism for the protective effect in stroke? Neurochem Res 2010; 35:1391–401
37. Soares LM, De Vry J, Steinbusch HWM, Milani H, Prickaerts J, Weffort de Oliveira RM: Rolipram improves cognition, reduces anxiety- and despair-like behaviors and impacts hippocampal neuroplasticity after transient global cerebral ischemia. Neuroscience 2016; 326:69–83
38. Takeichi T, Takarada-Iemata M, Hashida K, Sudo H, Okuda T, Kokame K, Hatano T, Takanashi M, Funabe S, Hattori N, Kitamura O, Kitao Y, Hori O: The effect of Ndrg2 expression on astroglial activation. Neurochem Int 2011; 59:21–7
39. Mitchelmore C, Büchmann-Møller S, Rask L, West MJ, Troncoso JC, Jensen NA: NDRG2: A novel Alzheimer's disease associated protein. Neurobiol Dis 2004; 16:48–58
40. Araya-Callís C, Hiemke C, Abumaria N, Flugge G: Chronic psychosocial stress and citalopram modulate the expression of the glial proteins GFAP and NDRG2 in the hippocampus. Psychopharmacology (Berl) 2012; 224:209–22
41. Herskowitz JH, Seyfried NT, Duong DM, Xia Q, Rees HD, Gearing M, Peng J, Lah JJ, Levey AI: Phosphoproteomic analysis reveals site-specific changes in GFAP and NDRG2 phosphorylation in frontotemporal lobar degeneration. J Proteome Res 2010; 9:6368–79

42. Takarada-Iemata M, Kezuka D, Takeichi T, Ikawa M, Hattori T, Kitao Y, Hori O: Deletion of N-myc downstream-regulated gene 2 attenuates reactive astrogliosis and inflammatory response in a mouse model of cortical stab injury. J Neurochem 2014; 130:374–87
43. Wang F, Gao Z, Li X, Li Y, Li X, Zhong H, Xu N, Cao F, Wang Q, Xiong L: NDRG2 is involved in anti-apoptosis induced by electroacupuncture pretreatment after focal cerebral ischemia in rats. Neurol Res 2013; 35:406–14
44. Xuan AG, Chen Y, Long DH, Zhang M, Ji WD, Zhang WJ, Liu JH, Hong LP, He XS, Chen WL: PPARα agonist fenofibrate ameliorates learning and memory deficits in rats following global cerebral ischemia. Mol Neurobiol 2015; 52:601–9
45. Webster CM, Kelly S, Koike MA, Chock VY, Giffard RG, Yenari MA: Inflammation and NFkappaB activation is decreased by hypothermia following global cerebral ischemia. Neurobiol Dis 2009; 33:301–12

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