



# Protective effects of Astragaloside IV against oxidative injury and apoptosis in cultured astrocytes by regulating Nrf2/JNK signaling

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Received: 9 December 2020 / Accepted: 19 March 2021 / Published online: 8 April 2021  
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## Abstract

Ischemic stroke is a worldwide complex brain disease that results in numerous disabilities and deaths. It leads to the deprivation of oxygen and glucose, which causes energy failure and neuronal death. The activation of astrocytes contributes to neuronal damage or repair after brain ischemia/reperfusion, although astrocytes get little attention as potential drug targets. This study investigated the protective effects of Astragaloside IV (AS-IV) on oxygen glucose deprivation/reoxygenation (OGD/R)-induced damage in rat primary cultured astrocytes and the underlying molecular mechanism. The results showed that compared with the control group, astrocytes under OGD/R exposure significantly decreased cell viability and increased the number of apoptotic cells, whereas AS-IV evidently protected the astrocytes against OGD/R-induced cell damage. In addition, low and medium concentrations of AS-IV can promote the increase of intracellular superoxide dismutase (SOD) level, as well as restored the morphological changes caused by OGD/R exposure. Supplementation with AS-IV after OGD/R exposure promoted the expression of oxidation and apoptosis indexes and further study demonstrated that AS-IV inhibited CXCR4 receptor and downregulated the activation of p-JNK/JNK pathway, which suppressed the expression of Bax/Bcl-2, and finally uprises Nrf2/Keap1 signaling. In conclusion, these findings revealed that AS-IV protected against OGD/R-induced astrocytes through inhibiting oxidative stress and apoptotic pathways.

**Keywords** Ischemic stroke · Deprivation/reoxygenation · Apoptosis indexes · Astrocytes · Oxidation

## Abbreviations

AS-IV Astragaloside IV  
OGD/R Oxygen glucose deprivation/reoxygenation  
CNS Central nervous system  
GFAP Glial fibrillary acidic protein

CXCR4 C-X-C motif chemokine receptor 4  
Keap1 Kelch-like ECH-associated protein-1  
Nrf2 (NF-E2)-related factor 2  
ERK Extracellular signal-regulated kinase  
p-ERK Phosphorylation-extracellular signal-regulated kinase  
JNK C-Jun N-terminal kinase  
p-JNK Phosphorylation-c-Jun N-terminal kinase  
Bcl-2 B-cell lymphoma-2  
Bax Bcl2-Associated X  
NIMO Nimodipine

Communicated by Bill J Yates.

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## Introduction

As a group of neurological diseases, ischemic stroke is a common disease leading to neurological deficits and cognitive impairments (Mori 2002), with the characteristics of high incidence, high disability rate, high mortality rate, high recurrence rate and many complications, it is one of the three major fatal diseases in the world (Basu et al. 2005). Because of this, the prevention and treatment of

cerebrovascular diseases has become an important topic in medical research in the world. The precise mechanisms of cerebral ischemia are still elusive, previous studies suggested mitochondrial dysfunction (Camilleri and Vassallo 2014), excitotoxicity, neuroinflammation (Sanchez-Guajardo et al. 2015) as well as oxidative stress (Cui et al. 2018) were all involved in this process.

Astrocytes represent an important fraction of glial cells in the central nervous system (CNS), widely distributed in the brain and spinal cord. The ratio of astrocytes to neurons in the mouse and rat cortex is 1:3, while the human cortex has an approximately 1:1 ratio of astrocytes: neuron (Azevedo et al. 2009; Nedergaard et al. 2003). They are the most populous glial subtype, essential for brain function, as we learn more about the biology of astrocytes (Lu et al. 2015), it is clear that they are essential for maintaining synapses, nerve repair, development, and plasticity. Under normal circumstances, astrocytes release glial transmitters and respond to cytokines, chemokines and growth factors, maintain metabolic balance and homeostasis in synapses. Therefore, the disruption of astrocyte biology may contribute to CNS dysfunction and pathology (Sofroniew and Vinters 2010). In acute neuroinflammation, such as ischemic stroke, these cells can react and change their morphology through long-term overexpression of glial fibrillary acidic protein (GFAP) (Bao et al. 2013; Benarroch 2005; Rossi et al. 2007). With the development of ischemic infarction, the homeostasis provided by astrocytes is affected, glutamate uptake decreases, energy metabolism slows down, active molecular exchange decreases and antioxidant activity decreases (Ben Haim et al. 2015). Despite these highlighted advances, until now, no effective treatment is available to improve brain repair and neurological recovery, glia-mediated ischemic injury and protection have received only limited attention.

Astragaloside IV (AS-IV), the primary pure saponin isolated from the root of *Astragalus membranaceus*, is an effective compound with distinct pharmacological effects. AS-IV has been reported to exert anti-aging and immunomodulatory effects, as well as anti-inflammatory and anti-oxidative neuroprotective activities (Chang et al. 2016; Jiang et al. 2019; Wen et al. 2018; Zhang and Deng 2019). Previously, AS-IV has been reported in vitro to reduce apoptosis and increase the resistance of dopamine neurons to neurotoxins (Chan et al. 2009). However, effects of AS-IV on glia in cerebral ischemia have received little attention.

Here we used cultured rat astrocytes to investigate the role of AS-IV in protecting against oxidative damage in vitro oxygen and glucose deprivation/reoxygenation (OGD/R) model. This research provides evidence of oxidative stress, anti-apoptosis, anti-inflammatory during hypoxia-reoxygenation that may participate in the damaging effects of ischemic insult to the central nervous system.

## Materials and methods

### Primary cell culture

Primary astrocytes were derived from 0 to 3 day postnatal Sprague–Dawley (SD) rats ( $n=6$ ) (Ji et al. 2011). Neonatal rats were purchased from the Animal Research Center of Zhejiang Chinese Medical University. Before starting the dissection procedure, T75 culture flasks were coated with 50  $\mu\text{g}/\text{mL}$  poly-L-lysine (PLL) and incubated in a 37 °C cell culture grade incubator for 4 h. Cortices were dissected and incubated with 20 U/mL papain for 20 min at 37 °C. The tissue was triturated and suspended in Dulbecco's modified Eagle's medium (DMEM, BI, Haemek, ISR). Cells were plated and maintained as described previously (Ji et al. 2011). When the cell density grew to 70% of the plate bottom, cells were transferred to poly-L-lysine (PLL)-coated plate and plated at a uniform density of  $2 \times 10^5$  cells/mL. In these cultures, approximately 95% of the cells were astrocytes based on immunocytochemical criteria, as shown by positive staining for glial fibrillary acidic protein (GFAP) (Supplemental Fig. 1).

### Cell treatment

Cerebral Cortexes were dissected from newborn SD rats and glial mixture cells were planted in T75 culture flasks. Change the medium every 3 days, after 7 days of culture, astrocytes were collected from glial culture by shaking off other glia cells at 250 rpm under 37 °C for 16–18 h. Astrocytes were then digested, collected, re-plated onto poly-L-lysine-coated plates/dishes and grown in DMEM medium supplemented with 10% fetal bovine serum (FBS). 24 h after cell adherence, astrocytes were given oxygen glucose deprivation/reoxygenation (OGD/R) and further cell treatment.

Astrocytes were washed twice with glucose-free DMEM and maintained in glucose-free DMEM. Then, the cells were placed in a 94% N<sub>2</sub>, 5% CO<sub>2</sub> and 1% O<sub>2</sub> model incubator at 37 °C, incubated for 1–4 h under simulated ischemic conditions while control cells were incubated in normal medium at 37 °C in a regular CO<sub>2</sub> (5%) incubator. After 4 h of OGD, aspirate the glucose-free medium, wash 1–2 times with HBSS, change to normal medium or drug containing normal medium, incubated at 37 °C in a regular CO<sub>2</sub> (5%) incubator for 24 h, and then perform subsequent experiments.

### Cell viability assay

Cell viability was determined by the Cell Counting Kit-8 (CCK-8, ZOMANBIO, Beijing, China). The astrocytes ( $5 \times 10^4$ ) were seeded in 96-well plates. After 1 day of

incubation, the OGD model was made for 1 h, 24 h of administration, 10  $\mu$ L of CCK-8 solution was added to each well and incubated at 37 °C for 1 h. The absorbance was measured at 450 nm with a SPECTRA max microplate reader.

### Immunofluorescence staining

The astrocytes were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS and permeabilized with 0.2% Triton X-100 for 10 min, blocked in 10% goat serum and 1% BSA for 1 h, then labeled with primary antibody (GFAP, 1:1000) overnight at 4 °C. Then cells were washed three times with PBS and were incubated with corresponding secondary antibody (1:1000) for 1 h at RT. The cultures were mounted using ProLong Gold Antifade Reagent with DAPI. The stained cells were observed using a fluorescent inverted live cell microscope (Carl Zeiss AG, Germany) and analyzed using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

### Quantification of apoptosis by flow cytometric analysis

FITC (fluorescein isothiocyanate) Annexin V Apoptosis Detection Kit I (BD Biosciences) was used to detect astrocyte apoptosis in this study. Wash cultured astrocytes twice with cold PBS and then resuspended cells in 1X Binding Buffer at a concentration of  $1 \times 10^6$  cells/mL. Transfer 100  $\mu$ L of the solution ( $1 \times 10^5$  cells) to a 5 ml culture tube. Add 5  $\mu$ L of FITC Annexin V and 5  $\mu$ L propidium iodide (PI). Gently vortex the cells and incubate for 15 min at RT in the dark. Add 400  $\mu$ L of 1X Binding Buffer to each tube. The cell suspensions were not sorted for pure astrocytes and directly analyzed by flow cytometry (Cytoflex S) within 1 h.

### Determination of SOD activity

The SOD activity of astrocytes was detected by WST-8 method. Cells were washed once in PBS at 4 °C, 100  $\mu$ L of the SOD sample preparation solution provided by the kit was added to fully lyse the cells, then centrifuged at 12,000 r/min for 5 min, the supernatant was taken as the sample to be tested. Samples were added to 96-well plates according to the kit method, incubated at 37 °C for 30 min, and recorded the absorbance at 450 nm with a SPECTRA max microplate reader.

### Quantitative real-time PCR (RT-qPCR)

TRIzol reagent (Invitrogen, USA) was used to extract total RNA from primary astrocytes of SD rats. RNA was reverse transcribed into cDNA using 5  $\times$  All-In-One RT Master Mix (abm, Richmond, CA). Then, real-time PCR was assessed

using Ultra SYBR Mixture (CW BIO, China) and ABI 7500 (ABI, USA). Experiments were performed in replicates. A control experiment was performed using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an internal reference index, the relative expression level was analyzed by the  $2^{-\Delta\Delta C_t}$  method. The primer sequences are shown in Table 1.

### Western blotting analysis

Cells were harvested and lysed with a cold SDS gel sample buffer containing protease inhibitors (Bimake). The samples were separated by 10% SDS-PAGE gel electrophoresis, and the protein electrophoresis was transferred to a PVDF membrane. The membranes were blocked with 5% skim milk for 1 h, then incubated overnight at 4 °C with the primary antibody, including Bcl-2 (1:1000), Bax (1:1000), CXCR4 (1:200), Keap1 (1:500), Nrf2 (1:500), ERK (1:500), phospho-ERK (1:500), JNK (1:500), phospho-JNK (1:500), and internal control  $\beta$ -actin (1:1000), all from Abcam (Cambridge, MA, USA). After incubating with horseradish peroxidase-conjugated secondary antibody, chemiluminescence reagents (ECL, Bio-Rad) were used to display immunoreactive bands. The membrane signals were digitally scanned, and the signals on the digital images were quantitated using AzueSpot analysis software.

### Statistical analysis

GraphPad Prism 8.0 was used to analyze the data. All data are shown as the mean  $\pm$  SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. *P* values < 0.05 were considered to indicate a statistically significant difference.

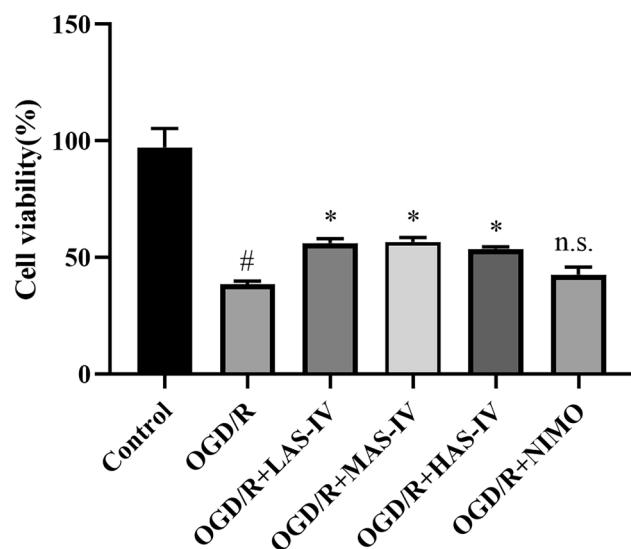
**Table 1** Primers used for reverse transcription-quantitative polymerase chain reaction

Gene	Primer sequence
<i>Gapdh</i>	F: 5'-GCAAGTTCAACGGCACAG-3' R: 5'-GCCAGTAGACTCCACGACAT-3'
<i>Sod1</i>	F: 5'-AAGCGGTGAACCAGTTGTGG-3' R: 5'-CAACATGCCTCTCTTCATCCG-3'
<i>Sod2</i>	F: 5'-ACGCGACCTACGTGAACAAT-3' R: 5'-GCCTCCAGCAACTCTCTTT-3'
<i>Sod3</i>	F: 5'-AGAGCTTGTCAGGTGTGGAAC-3' R: 5'-TCAAGCCGGTCTGCTAAGTC-3'
<i>Bcl-2</i>	F: 5'-GGCCTTCTTTGAGTTCGGTG-3' R: 5'-ATATAGTTCACAAAGGCATCCCAG-3'
<i>Bax</i>	F: 5'-GAGCTGCAGAGGATGATTGCTG-3' R: 5'-TCTGATCAGCTCGGGCACTT-3'
<i>Cxcr4</i>	F: 5'-TGCCATGGAAATATACACTTCGG-3' R: 5'-TGCCCACTATGCCAGTCAAG

## Results

### Effects of AS-IV on primary astrocyte injury induced by OGD/R

To study the protective of AS-IV, after 4 h OGD, primary astrocytes received hypoxia-induced reperfusion with different concentrations of AS-IV, the low-dose AS-IV group (LAS-IV, 16  $\mu$ M), the medium-dose group (MAS-IV, 32  $\mu$ M), the high-dose AS-IV group (HAS-IV, 64  $\mu$ M), and the nimodipine group (Nimo, 20  $\mu$ M). Nimodipine is a dihydropyridine calcium channel antagonist, which can enter the central nervous system through the blood–brain barrier, it has been reported that nimodipine reduced the damage of transient focal cerebral ischemia in vivo, and significantly increased cell viability, inhibited autophagy in OGD/R-treated in vitro model (Li et al. 2019). In this study, nimodipine was used as a positive drug for comparison. The cell viability rate was detected by CCK-8 method. Results showed that the viability of astrocytes in the OGD/R group ( $58.67\% \pm 8.270\%$ ,  $n=4$ ) was significantly decreased compared with the control group ( $P < 0.001$ ). Furthermore, the viability of astrocytes in LAS-IV ( $17.59\% \pm 2.387\%$ ,  $n=4$ ), MAS-IV ( $18.14\% \pm 2.345\%$ ,  $n=4$ ) and HAS-IV ( $15.11\% \pm 1.683\%$ ,  $n=4$ ) groups were markedly increased compared with the OGD/R group ( $P < 0.05$ ). This indicated that AS-IV treatment can alleviate OGD/R injuries (Fig. 1).



**Fig. 1** Protective effect of AS-IV on astrocytes induced by OGD. Cell viability was measured by CCK-8 assay. The results represent the mean  $\pm$  SEM. of at least three independent experiments, and are expressed as a percentage of the control. # $P < 0.001$ , compared with control; \* $P < 0.05$ , n.s., compared with the OGD/R group

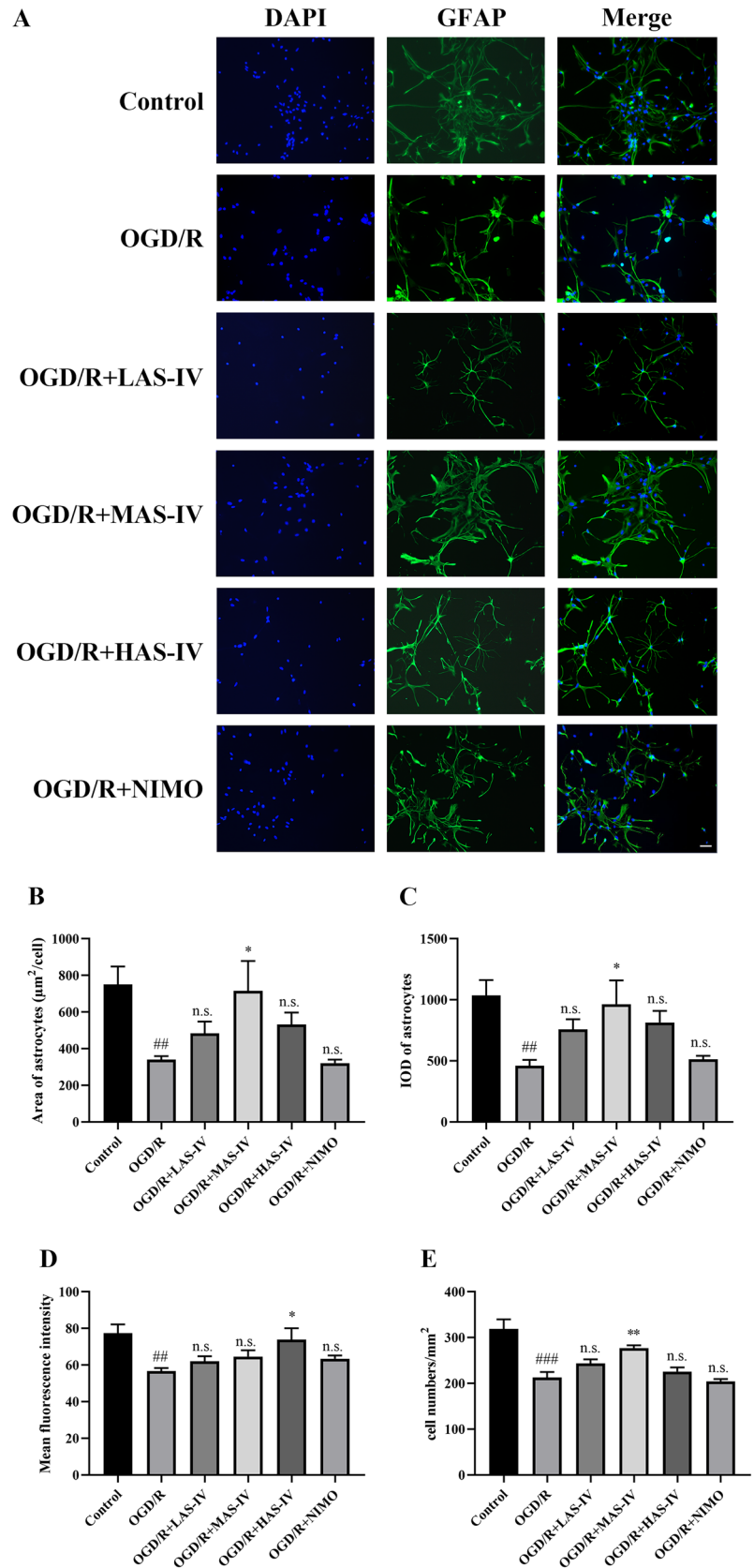
### The effect of AS-IV on restoring the morphology of astrocytes

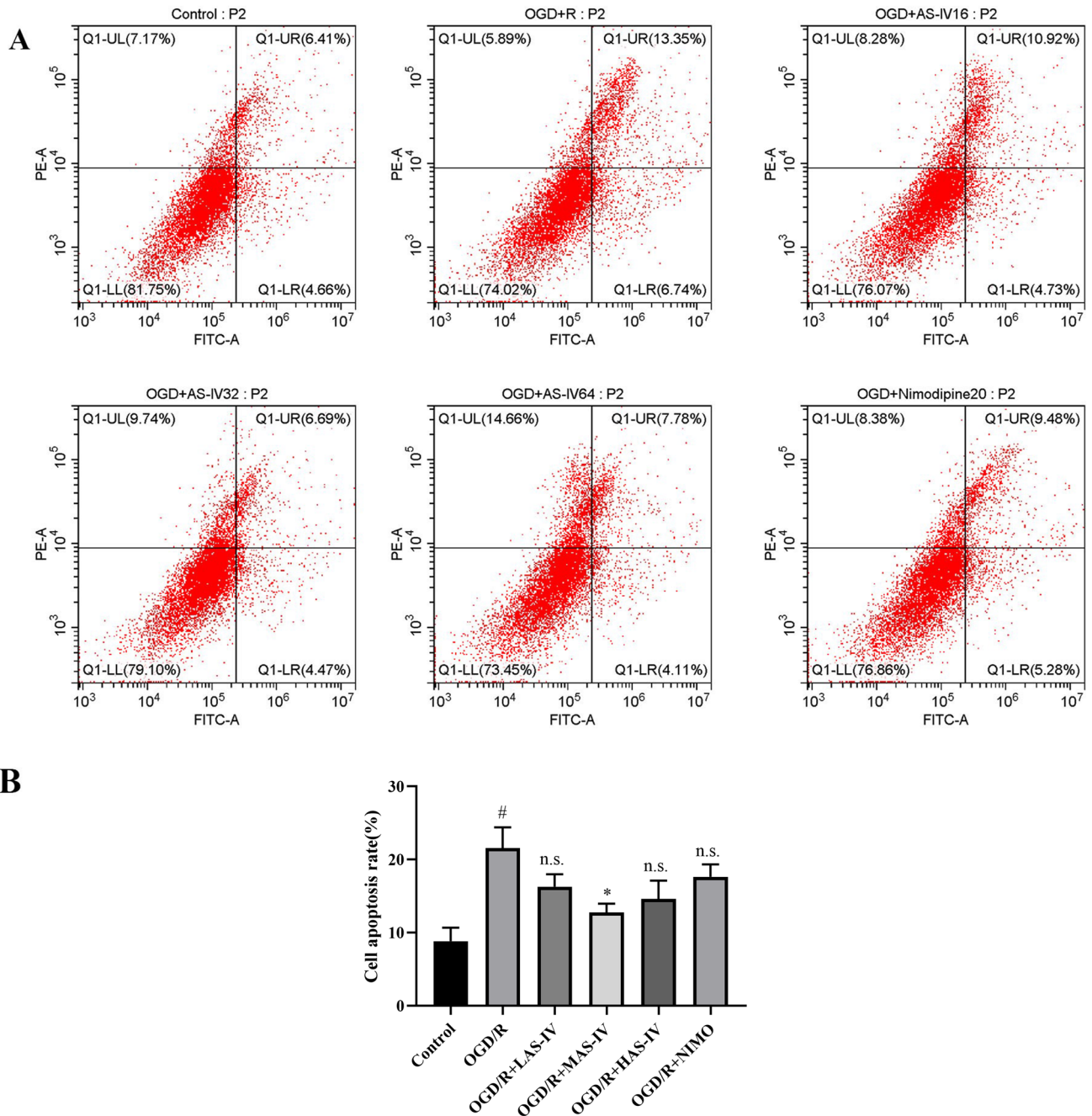
As previous reports, the morphology of astrocytes changed significantly after OGD/R, the activated astrocytes participate in a series of inflammation reactions such as proinflammatory cytokine secretion and immune cell attraction (Yin et al. 2018). To investigate whether AS-IV could invert the activation of astrocytes after OGD/R, primary mature astrocytes were identified by GFAP immunofluorescence and stained with DAPI to estimate the number of cells. After administering the cell model, the outline and appearance of astrocytes were observed to be thinner, and the surface area of astrocytes ( $\mu\text{m}^2/\text{cell}$ ) was significantly decreased compared with the control group ( $410.7 \pm 99.60$ , Fig. 2b,  $n=8$ ). In addition, the integrated optical density (IOD) and mean fluorescence intensity of glial fibrillary acidic protein (GFAP) were significantly decreased in the OGD group compared with the control group ( $574.9 \pm 134.8$ ;  $20.61 \pm 5.155$ , Fig. 2c, d,  $n=8$ ). However, the morphology of astrocytes was markedly improved in the OGD/R + AS-IV groups compared with the OGD/R group (Fig. 2a). Moreover, the area of astrocytes significantly increased to  $376.0 \pm 162.9$  (Fig. 2b,  $n=8$ ), IOD of GFAP increased significantly to  $503.0 \pm 200.5$  (Fig. 2c,  $n=8$ ), and the mean fluorescence intensity of GFAP increased significantly to  $17.2 \pm 6.438$  (Fig. 2d,  $n=8$ ) following treatment with MAS-IV and HAS-IV compared with the OGD group. The OGD/R also caused an obvious reduction of cell density ( $212.9 \pm 11.99$  cells/ $\text{mm}^2$  in OGD/R group versus  $319.3 \pm 20.63$  cells/ $\text{mm}^2$  in control, Fig. 2e,  $n=8$ ), while MAS-IV treatment could restore the cell density ( $277.1 \pm 5.962$  cells/ $\text{mm}^2$  in MAS-IV group versus  $212.9 \pm 11.99$  cells/ $\text{mm}^2$  in OGD/R group, Fig. 2e,  $n=8$ ). The above results indicated AS-IV could restore OGD/R-induced morphological changes and quantity decrease of astrocytes.

### The effect of AS-IV on apoptosis of astrocytes

OGD/R injury is closely associated with the stimulation of apoptosis. To further investigate whether AS-IV could protect astrocytes from apoptosis after OGD/R injuries. The primary astrocytes in the AS-IV and Nimodipine groups were treated with different concentrations (16, 32, 64, and 20  $\mu$ M) after reoxygenation (OGD, 4 h), and analyzed by flow cytometry 24 h later. Compared with the normal group, the apoptosis rate in the OGD/R group was significantly increased by 12.72% ( $n=3$ , Fig. 3). Compared with the

**Fig. 2** AS-IV reduced the OGD/R-induced morphological change of astrocytes. **a** All images were stained with DAPI (blue) for nuclear staining, and glial fibrillary acidic protein (GFAP, green) was used to observe the activation of astrocytes. Scale bars = 50  $\mu\text{m}$ . **b** Area of astrocytes ( $\mu\text{m}^2/\text{cell}$ ) in each group. **c** IOD of astrocytes in each group. **d** Mean fluorescence intensity in each group. **e** Cell numbers in each group. ##  $P < 0.01$ , compared with control; \*  $P < 0.05$ , n.s., compared with the OGD/R group. IOD integrated optical density





**Fig. 3** AS-IV attenuates OGD/R-induced apoptosis of mature primary astrocytes. The astrocytes were analyzed by flow cytometry after treatment. **a** Hippocampal neurons were stained with annexin V-FITC and PI. **b** Percentages of apoptotic astrocytes in the control

cultures and each separate experimental culture were determined. Data are presented as the means  $\pm$  SEM of values from triplicate independent experiments. # $P < 0.05$ , compared with control; \* $P < 0.05$ , n.s., compared with the OGD/R group

OGD/R group, apoptosis rate in all concentrations of AS-IV and nimodipine groups showed a declining tendency, and the MAS-IV group could significantly decrease the apoptosis rate (Fig. 3b). These results suggested that AS-IV inhibited apoptosis and necrosis of OGD/R-induced astrocytes.

### The effect of AS-IV on expression of apoptosis indexes

B-cell lymphoma 2 (Bcl-2) family proteins play an important role in inducing apoptosis, and the ratio of Bcl2-Associated X (Bax)/Bcl-2 is closely related to apoptosis. To further verify the effects of AS-IV on apoptosis induced by OGD exposure, primary astrocytes were cultured and OGD/R-induced for 4 h, then reoxygenated with AS-IV and nimodipine for 24 h, then the mRNA and protein levels of Bcl-2 and Bax were analyzed by real-time quantitative PCR and Western blotting. Compared to the control group, mRNA expression of apoptotic factors was significantly decreased in the OGD/R group with each index, especially the anti-apoptotic factor Bcl-2 in the LAS-IV group was significantly increased (Fig. 4b). The apoptotic factors mRNA expression of the other administration groups slightly increased compared with the OGD/R group (Fig. 4a, b). Western blotting showed a significant increase of Bax in the OGD/R group compared to the control group, while MAS-IV and HAS-IV treatment remarkably reduced Bax expression after OGD/R induction (Fig. 4c, d). In contrast to Bax, there is a reverse expression pattern of Bcl-2 in AS-IV-treated OGD/R experiment, as an evident decrease ( $0.4575 \pm 0.0923$ ) after OGD/R introduction, and an obvious increase in LAS-IV ( $0.4387 \pm 0.1553$ ) and MAS-IV ( $0.6360 \pm 0.1706$ ) groups (Fig. 4c, e,  $n = 7$ ). The ratio of Bax/Bcl-2 in the OGD/R group increased ( $0.8106 \pm 0.2854$ ) compared with the control (Fig. 4c, f). However, the ratio of Bax/Bcl-2 decreased using AS-IV (Fig. 4c, f,  $n = 6$ ). Compared with the OGD/R group, MAS-IV ( $0.8819 \pm 0.3364$ ) and HAS-IV ( $0.8579 \pm 0.2650$ ) groups markedly decreased its ratio (Fig. 4c, f,  $n = 6$ ).

### AS-IV increases SOD activity and SOD mRNA expression in astrocytes

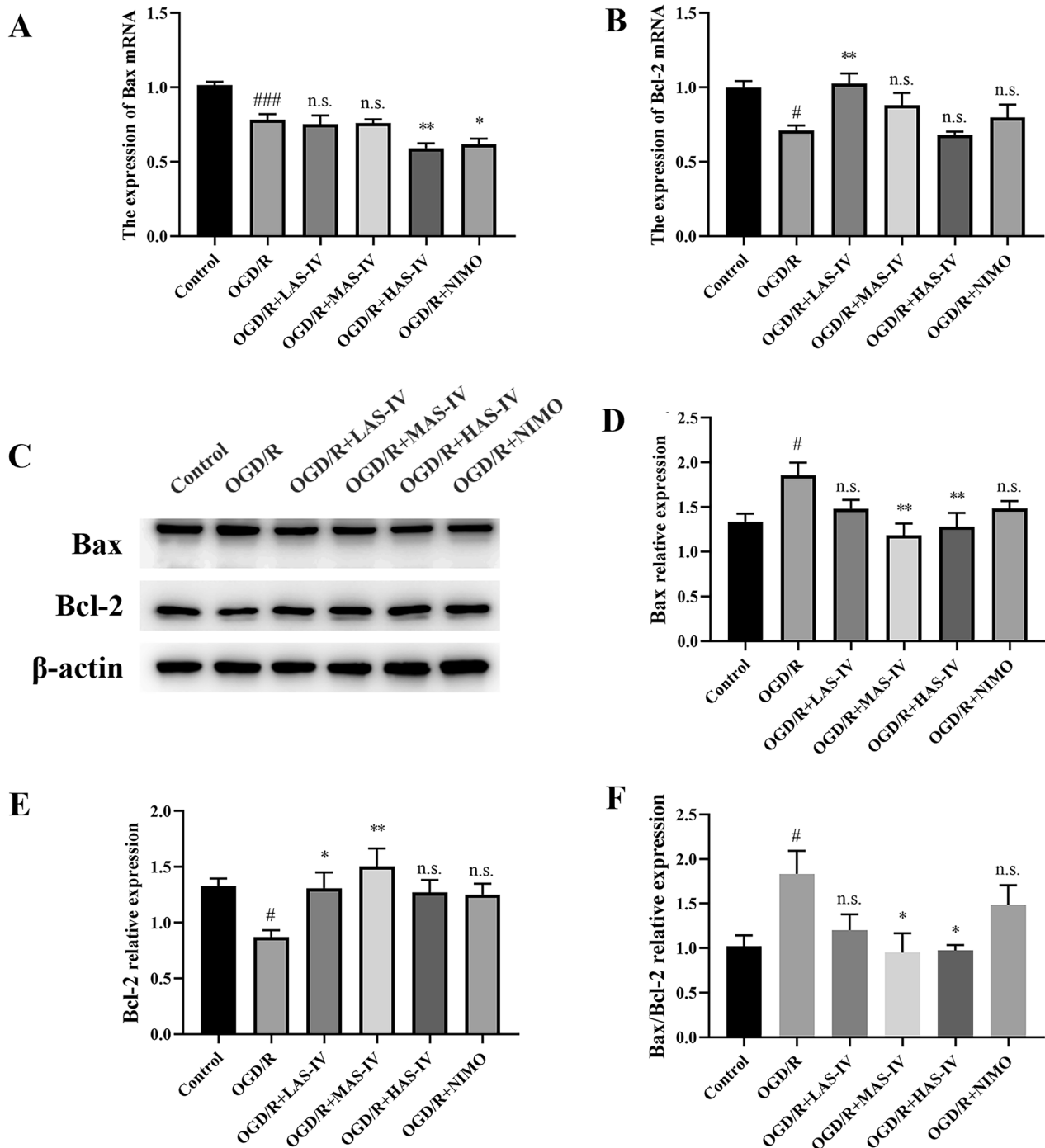
The generation of reactive oxygen species during cerebral I/R is widely regarded as the initial step after stroke and much associated with apoptosis. One of the most important antioxidant enzymes is superoxide dismutase (SOD), including CuSOD, ZnSOD and MnSOD, facilitating the dismutation of superoxide radicals to generate  $H_2O_2$ ,

which is further removed by other peroxidase enzymes. Therefore, we detected the level of SOD enzyme activity and mRNA expression in astrocytes treated with AS-IV after OGD/R. The results showed that the activity of SOD in the OGD/R group was lower than that in the control group (Fig. 5a). Compared with the OGD/R group, SOD activity increased in HAS-IV and nimodipine groups, and significantly increased in LAS-IV and MAS-IV groups (Fig. 5a).

Next, we evaluate the mRNA expression of SOD enzymes using real-time quantitative PCR. Results showed that the oxidative factor in the OGD/R group of SOD1 was markedly lower than that of the control (Fig. 5b). The OGD/R group of SOD2 and SOD3 also had a decreasing trend (Fig. 5c, d). Compared with the OGD/R group, the oxidation factor in other administration groups was all higher, especially SOD2 expression of MAS-IV and SOD3 expression of LAS-IV were significantly increased (Fig. 5c, d). These results indicated that AS-IV can increase the SOD activity and mRNA levels in astrocytes induced by OGD/R and implied that AS-IV might be involved in the protective effect of OGD/R-induced oxidative damage on astrocytes.

### The effect of AS-IV on the expression of regulatory factors related to oxidative stress

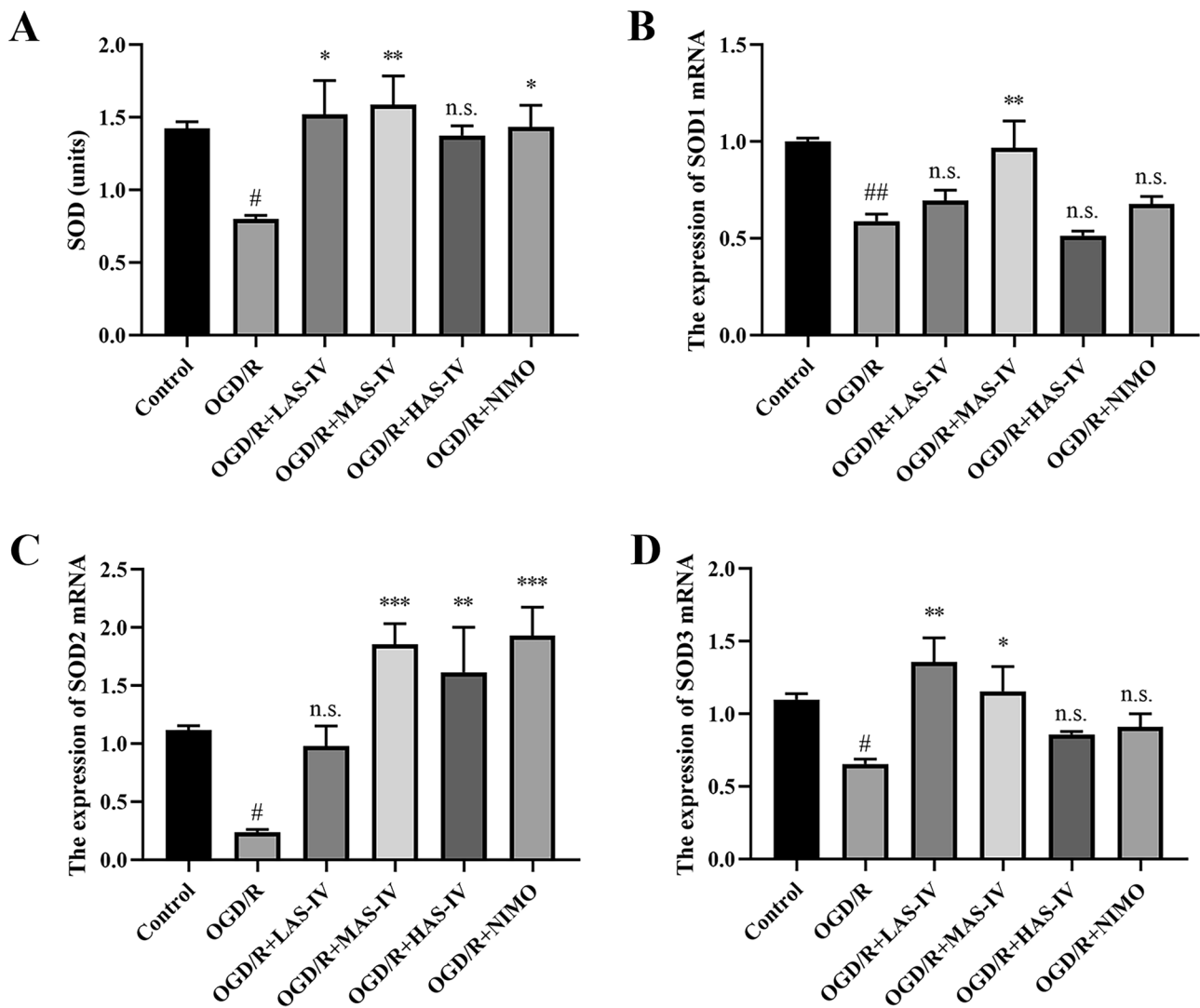
As an important regulator of cellular oxidative stress, Keap1/Nrf2 expression was then investigated to evaluate the effect of AS-IV on the antioxidative ability of cells induced by OGD/R. Compared with the control group, the expression of Keap1 in the OGD/R group was significantly increased by 51.4% (Fig. 6a, b,  $n = 9$ ). Compared with the OGD/R group, the drug administration group could reduce the expression of Keap1, and the MAS-IV ( $0.2904 \pm 0.1215$ ) group had a significant reduction of Keap1 expression (Fig. 6b,  $n = 9$ ). It was reported that once exposed to stimuli and pharmacological inducers, Nrf2 is separated from Keap1, translocated to the nucleus and combined with antioxidant response elements (AREs), further resulting in the expression of diverse genes with biological activity as antioxidants, such as SOD enzymes (Chen et al. 2017). Compared with the control group, the expression of Nrf2 in the OGD/R group was significantly decreased by 31.3% (Fig. 6a, c,  $n = 3$ ). Compared with the OGD/R group, the drug administration group could increase the expression of Nrf2, and the LAS-IV ( $0.2993 \pm 0.0537$ ) group had a significant increase of Nrf2 expression (Fig. 6c,  $n = 3$ ). Our results suggested that AS-IV regulates SOD expression through Keap1/Nrf2 pathways.



**Fig. 4** AS-IV promotes the increase of apoptosis factors OGD/R-induced at the level of mRNA and effect of AS-IV on the expression of Bcl-2 family-associated proteins in primary astrocytes induced by OGD exposure. **a** The mRNA expression of Bax after OGD/R treatment. **b** The mRNA expression of Bcl-2 after OGD/R treatment. **c** Bax and Bcl-2 were detected by specific antibodies, respectively, whereas  $\beta$ -actin was detected as the internal control. **d** Quantification of Bax

protein expression after OGD/R treatment. **e** Quantification of Bcl-2 protein expression after OGD/R treatment. **f** The Western blot were quantified and expressed as the ratio of Bax/Bcl-2. Representative blot is shown at least from three independent experiments with similar results. The results represent the mean  $\pm$  SEM. of at least three independent experiments. # $P < 0.05$ , ### $P < 0.001$ , compared with control; \* $P < 0.05$ , \*\* $P < 0.01$ , n.s., compared with the OGD/R group





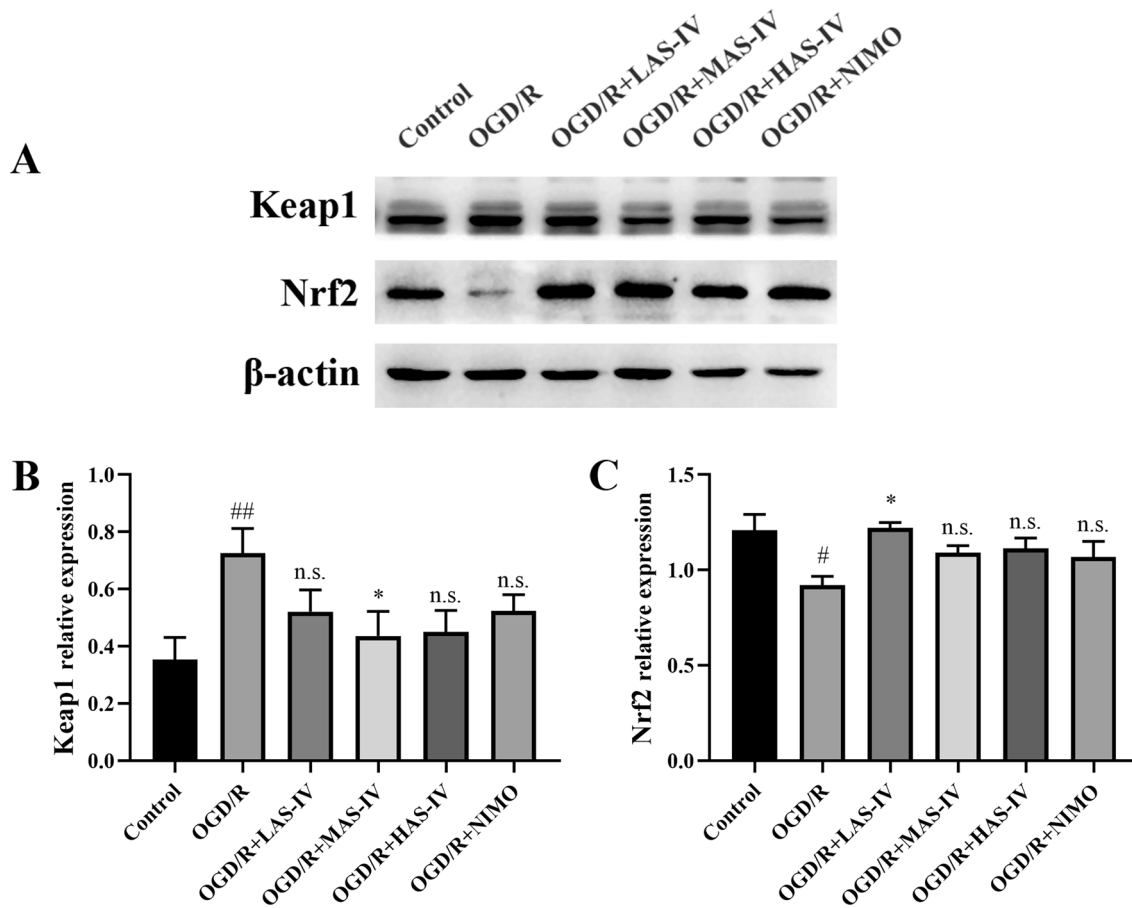
**Fig. 5** The effect of as-iv on the activity of SOD1 in astrocytes induced by OGD and the increase of oxidative factors induced by OGD/R at the mRNA level. The absorbance of 450 nm was measured by enzyme labeling instrument, and the activity of SOD1 enzyme in each group was calculated according to the instructions of the kit. The numerical values are obtained from three parallel experiments.

**a** SOD activity quantification after OGD/R treatment. **b** mRNA expression of SOD1 after OGD/R treatment. **c** mRNA expression of SOD2 after OGD/R treatment. **d** The mRNA expression of SOD3 after OGD/R treatment. #*P* < 0.05, ##*P* < 0.01, compared with control; \**P* < 0.05, n.s., compared with the OGD/R group. SOD superoxide dismutase

### Effects of AS-IV on the expression of MAPK pathway-related proteins

Extracellular regulated protein kinases (ERK) and c-Jun N-terminal kinase (JNK) are members of mitogen-activated protein kinase (MAPK) cascades. They are activated by oxidative stress to adjust the intracellular redox status. They are also common up stream pathway proteins that can lead to apoptotic response signaling, such as pro-apoptotic proteins Bax and Bcl-Xs (Wang

et al. 2019), to confirm the role of ERK/JNK as a factor involved with the AS-IV protective effect, we analyzed the protein and phosphorylation protein levels in astrocyte OGD/R model as previous described. OGD/R induction significantly (Fig. 7a, b, *n* = 3) increased ( $0.4132 \pm 0.1565$ ) phosphor-JNK (p-JNK)/JNK ratio, compared to the control group. Alternatively, p-JNK/JNK ratios were markedly reduced ( $0.4162 \pm 0.1594$ ;  $0.3743 \pm 0.1573$ ;  $0.3671 \pm 0.1593$ ) in AS-IV groups (Fig. 7a, b, *n* = 3) compared to the OGD/R group, while



**Fig. 6** The effect of AS-IV on the expression of regulatory factors related to oxidative stress in primary astrocytes induced by OGD exposure. **a** The Western blot were quantified and expressed as the signal intensity ratio of Keap1 and Nrf2, and  $\beta$ -actin was detected as the internal control. Representative blot is shown at least from three independent experiments with similar results. **b** Quantification of

Keap1 expression level after OGD/R treatment. **c** Quantification of Nrf2 expression level after OGD/R treatment. The results represent the mean  $\pm$  SEM. of at least three independent experiments. # $P < 0.05$ , ## $P < 0.01$ , compared with control; \* $P < 0.05$ , n.s., compared with the OGD/R group

there was no obvious change in phosphor-ERK (p-ERK)/ERK ratios (Fig. 7a, c). Therefore, AS-IV may inhibit apoptosis by downregulating JNK signaling.

Chemokines, a class of small proteins acting via cell-surface G protein-coupled receptors, have been increasingly shown to modulate numerous of biological responses, including enzyme secretion, cell adhesion and cytotoxicity, etc. (Chuang et al. 2016). Stromal cell-derived factor 1 (CXCL12/SDF-1) belongs to the CXC subfamily

of chemokine and exerts its effects via the C-X-C motif chemokine receptor 4 (CXCR4) receptor, which is widely expressed in neurons and astrocytes. Previous studies showed binding of CXCL12 and CXCR4 are able to regulate cell survival by activating multiple signaling pathways, such as ERK1/2, p38, MAPK, PLC/MAPK, and JNK (Zhou et al. 2019), so we examined the mRNA and protein levels in astrocyte OGD/R models to confirm whether AS-IV contribute to regulating CXCR4. RT-qPCR and immunoblot

experiments showed significant downregulation of CXCR4 in AS-IV groups (Fig. 7a, d, e), indicating CXCL12/CXCR4 probably as upstream factors of JNK.

## Discussion

At present, the onset age of ischemic stroke is getting younger, with the characteristics of high incidence, high disability rate and high mortality. During the occurrence of ischemic stroke, the oxygen supply and energy source of neurons in the brain are blocked due to blockage of cerebral arteries, resulting in neuronal damage. The existing treatments are still limited. This fact promotes a comprehensive study of cerebral ischemia to find new treatments and targets. It has been reported that AS-IV has the pharmacological activities of anti-inflammation, anti-oxidation, anti-virus, anti-apoptosis, and immune regulation (Bicalho et al. 2002). In this study, we investigated the effects of AS-IV on astrocyte OGD/R model and discovered that AS-IV could protect against OGD/R injury through the induction of Nrf2 via the MAPK pathway.

Cerebral ischemia is a complex pathological process that includes the generation of oxidative stress, inflammation, excitatory neurotoxicity, cell death, and regeneration. Oxidative stress has long been recognized to be responsible for the occurrence and progression of ischemia. Therefore, it is important to explore if AS-IV can reduce oxidative stress. Our results clearly indicated that AS-IV increased expression of antioxidant enzymes SOD2 and SOD3 (Fig. 5b), which are considered important in eliminating ROS and vital for the cellular defense mechanism toward oxidative stress (Rodrigues et al. 2018). In this study, treatment with AS-IV remarkably induced increased protein level of Nrf2, a coordinator for many signaling pathways, mitigates oxidative stress-induced cell injury by mediating the expression of various genes (Luo et al. 2020). Our results suggested that AS-IV clearly upregulated the Keap1-Nrf2 signaling pathway and since changes of SOD gene expression were consistent with the Nrf2 gene, it suggest that Nrf2 is required for inducing antioxidant genes in our OGD/R model. We should further test more antioxidant enzyme expression, including HO-1, NQO1, GCLM, and GCLC (Ge et al. 2017).

Apoptosis is a major mechanism of cell death and is characterized by a series of processes that are involved in the activation of a cascade of molecular events leading to cell death. Numerous studies have shown that oxidative stress such as ROS and a decreased efficiency of antioxidant defenses, can trigger cell death signaling cascades (Rodrigo

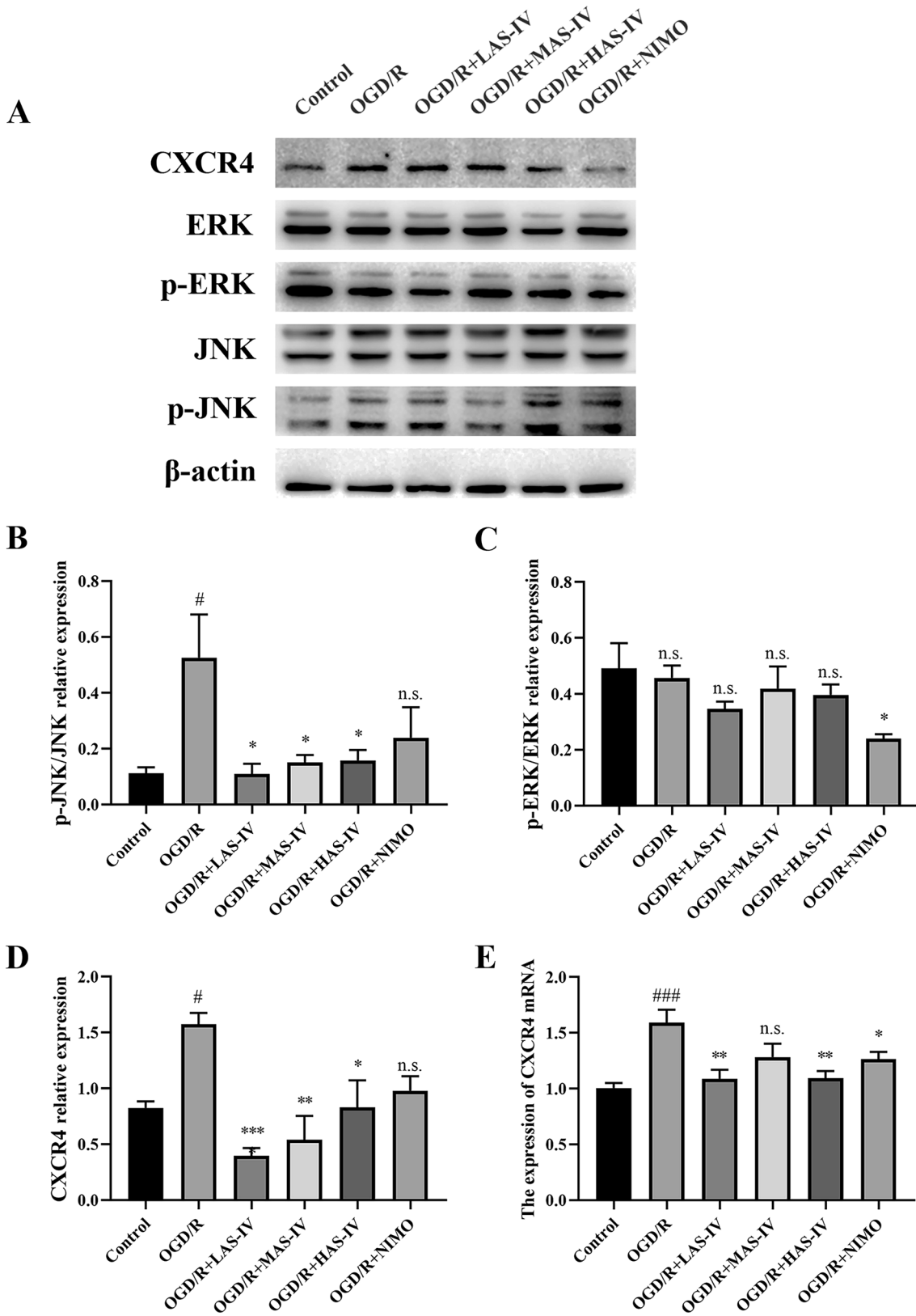
et al. 2013). The Bcl-2 family and Bax are both important regulators of the induced mitochondrial pathway of apoptosis. Bcl-2 exerts a pro-survival effect in response to apoptotic stimuli through the inhibition of mitochondrial cytochrome *c* release, while Bax localizes from the cytosol to the mitochondrial membrane and increases the permeability of the membrane, which releases cytochrome *c* from the mitochondria (Lauterwasser et al. 2019). Consistent with these findings, our results showed that OGD/R-induced downregulation of Bcl-2 and upregulation of Bax, which can be reversed by AS-IV treatments (Fig. 4c, f).

MAPKs represent a highly conserved subfamily of serine/threonine protein kinases, which are important mediators of signal transduction from the cell cytoplasm to the nucleus. ERK1/2, JNK and p38MAPK are three well-characterized MAPK subfamilies. It has been reported that activation of MAPK signaling, specifically p38MAPK and JNK, is relevant to the regulation of neuronal apoptosis during I/R injury (Feng et al. 2018). Our results show that AS-IV suppress oxidative stress and inhibits the JNK pathways (Fig. 7a, b), and that inhibition of JNK regulates Bax/Bcl-2 expression and attenuates apoptosis. However, the ERK pathway had no effect on OGD/R-induced astrocyte apoptosis (Fig. 7a, c).

Astrocytes, the most abundant glial cell type in the brain, provide metabolic and trophic support to neurons and modulate synaptic activity under normal physiological conditions. Although neurons are more susceptible to ischemia, astrocytes also undergo damage after ischemic events. Astrocyte impairment contributes to the decrease of neuronal viability and functionality under ischemic conditions. Previous studies successively revealing the post-stroke role of astrocytes remained insufficient owing to the diversity of astrocyte functions. New evidence was still needed. Investigation of drugs that reduce apoptosis through the maintenance of astrocytes functionality could be an interesting neuroprotective strategy against several neurological diseases where oxidative stress is a key element.

Unfortunately, the regulatory mechanism between CXCL12/CXCR4 and JNK remains unexplained in this study. The mitogen-activated protein kinase 1 (MEKK1) might be one of the most direct regulating factors (Lin et al. 2018). However, the whole upstream signal pathway should be considered deliberately if we want to reveal the underlying mechanism of the regulating effect of AS-IV on JNK inhibition. This is one of the directions that should be focused upon in the future.

In conclusion, our results showed that AS-IV as a neuroprotective agent for OGD/R-induced oxidation injury and



**Fig. 7** Effects of AS-IV on the expression of MAPK pathway-related proteins induced by OGD/R in primary astrocytes. **a** Activation of ERK and JNK was examined by the level of phosphorylation with Western blot. Total ERK and JNK were assessed as internal controls for protein concentrations loaded. The expression level of CXCR4 was detected with immunoblotting, and  $\beta$ -actin was detected as the internal control. Representative blot is shown at least from three independent experiments with similar results. **b** The Western blot were quantified and expressed as the ratio of p-JNK/JNK after OGD/R induction. **c** The Western blot were quantified and expressed as the ratio of p-ERK/ERK after OGD/R induction. **d** Quantification of CXCR4 expression after OGD/R induction. **e** The mRNA expression of CXCR4 after OGD/R induction. The results represent the mean  $\pm$  SEM. of at least three independent experiments. # $P < 0.05$ , ### $P < 0.001$ , compared with the control group; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , n.s., compared with the OGD/R group. ERK extracellular regulated protein kinases, JNK C-Jun kinase enzyme; CXCR4 chemokine (C-X-C Motif) receptor 4

apoptosis by upregulating Keap1-Nrf2 signaling through repressing the activation of CXCR4/JNK pathway.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00221-021-06096-7>.

**Author contributions** JY: conceptualization, methodology, data curation, and writing—original draft. CS: conceptualization, methodology, data curation, writing—original draft, and funding acquisition. WL: methodology, data curation, and writing. HW: supervision, project administration, resources, funding acquisition, writing—review and editing. YH: supervision, project administration, resources, funding acquisition, writing—review and editing. JY: supervision, project administration, resources, funding acquisition, writing—review and editing.

**Funding** This project was supported by the National Natural Science Foundation of China (nos. 81973560, and 81630105). National Key R and D Program of China (no. 2017YFC1700400 and 2017YFC1700403), and Natural Science Foundation of Zhejiang Province (LZ18H270001, and Y20H280055). Thanks to the technical support from the Public Platform of Medical Research Center, Academy of Chinese Medical Science, Zhejiang Chinese Medical University.

**Availability of data and materials** All data generated and/or analyzed during this study are included in this article.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics approval and consent to participate** The animals used in our study were treated in accordance with protocols approved by the Animal Ethics Committee of Zhejiang Chinese Medical University.

**Consent for publication** Not applicable.

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