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Gastrodin Inhibits Inflammasome Through the STAT3 Signal Pathways in TNA2 Astrocytes and Reactive Astrocytes in Experimentally Induced Cerebral Ischemia in Rats

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Abstract

This study was aimed to determine Gastrodin (GAS) and its underlying signaling pathway involved in suppression of inflammasome specifically in reactive astrocytes that are featured prominently in different neurological conditions or diseases including cerebral ischemia. For this purpose, TNA2 astrocytes in cultures were exposed to oxygen–glucose–deprivation (OGD) mimicking hypoxic cerebral ischemia. Separately, TNA2 cells were pretreated with GAS prior to OGD exposure. Additionally, Stattic, an inhibitor of STAT3 signaling pathway, was used to ascertain its involvement in regulating inflammasome in astrocytes exposed to OGD. In parallel to the above, adult rats subjected to middle cerebral artery occlusion (MCAO) with or without GAS pretreatment were sacrificed at different time points to determine the effects of GAS on astrocyte inflammasome. TNA2 astrocytes in different treatments as well as reactive astrocytes in MCAO were processed for immunofluorescence labeling and Western blot analysis for various protein markers. In the latter, protein expression levels of p-STAT3, NLRP3, and NLRC4 were markedly increased in TNA2 astrocytes exposed to OGD. Remarkably, the expression levels of these biomarkers were significantly suppressed by GAS. Of note, GAS especially at dose 20 μM inhibited NLRP3 and NLRC4 expression levels most substantially. Moreover, GAS inhibited the downstream proteins caspase-1 and IL-18. Concomitantly, GAS significantly suppressed the expression of STAT3 and NF-κB signaling pathway. It is noteworthy that Stattic at dose 100 μM inhibited STAT3 pathway and NF-κB activation in TNA2 astrocytes, an effect that was shared by GAS. In MCAO, GAS was found to effectively attenuate p-STAT3 immunofluorescence intensity in reactive astrocytes. Arising from the above, it is concluded that GAS is anti-inflammatory as it effectively suppresses inflammasome in OGD-stimulated astrocytes as well as in reactive astrocytes in MCAO via STAT3 and NF-κB signaling expression coupled with decreased expression of caspase-1 and IL-18.

Keywords Gastrodin · OGD-astrocytes · Anti-inflammatory · NLRP3 · NLRC4 · STAT3 signaling · Cerebral ischemia

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Abbreviations

GAS	Gastrodin
OGD	Oxygen glucose deprivation
MCAO	Middle cerebral artery occlusion
STAT3	Signal transducers and activators of transcription
NLRP3	Nod-like receptor protein-3
NLRC4	NLR Family CARD Domain Containing 4
NF-κB	Nuclear factor κB
GFAP	Glial fibrillary acidic protein
DMEM	Dulbecco's modified Eagle's medium
TBS	Tris-buffered saline
FBS	Fetal bovine serum
PVDF	Polyvinylidene fluoride
PBS	Phosphate-buffered saline

Introduction

It is well recognized that cerebral ischemia is a major global health challenge as it can lead to permanent disability and mortality. Indeed, cerebral ischemia is a leading cause of arteriosclerosis and thrombosis as it causes transient or permanent occlusion of the brain arteries and ultimately deprivation of glucose/oxygen in brain cells (Fisher and Saver 2015). It is conceivable that this would result in cellular dysfunction and inflammation. Current treatments for ischemic stroke are limited in view of the complex molecular mechanisms involved in the neuropathology (Barrett et al. 2015). Administration of the thrombolytic agent tissue plasminogen activator (tPA) has been a therapeutic treatment of choice, but this has many shortcomings and limitations including the potential risk of hemorrhagic transformation and the narrow therapeutic window and efficacy (Tan et al. 2014). Thus, search for more effective therapeutic approaches for cerebral ischemia is desirable even though the underlying biochemical and molecular mechanisms responsible for the brain damage suffering from ischemic insult have remained to be fully explored and elucidated.

There is increasing evidence in recent years suggesting that inflammation is a major factor contributing to the pathogenic process in cerebral ischemia (Jin et al. 2010). It is well documented that Toll-like receptors (TLRs) are membrane receptors responsible for pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). It has been reported that TLR activation leads to expression of a wide range of inflammatory genes (Dinarello 1998). In this connection, the proinflammatory cytokine interleukin IL-1β is transcriptionally induced as a precursor protein after the activation of TLR signaling in inflammatory cells such as macrophages. The process requires the assembly of the cytoplasmic multiprotein complex referred to as inflammasome (Martinon and Tschopp 2007). Concomitantly, the innate sensors including Nod-like receptor, pyrin containing 3 (NLRP3), and NLR Family CARD Domain Containing 4 (NLRC4) are activated by diverse pathogen/danger-associated molecular patterns so as to regulate host cellular responses to infection and injury. It has been reported that inflammasome is activated at the early stage of ischemic stroke (Fann et al. 2013). The activation of NLRP3 and NLRC4 inflammasome by some factors including adenosine triphosphate (ATP) along with others causes the maturation of caspase-1 and the cleavage and production of interleukin-1β and interleukin-18 (Schroder and Tschopp 2010). It is well documented that neuroinflammation may be mediated by microglia and astrocytes (Dong and Benveniste 2001) and extensive studies have shown that the inflammasome is involved in neurological disorders (Halle et al. 2008); yet surprisingly, expression and roles of NLRP3 and NLRC4

in reactive astrocytes which are featured prominently in tissue repair and reconstruction in cerebral ischemia have not been fully investigated and clarified.

Gastrodin (GAS) is an active compound derived from the orchidaceae family. It has been widely used for thousands of years in clinic in China. Pharmacological studies indicate that GAS has analgesic, improves microcirculation and general circulatory functions (Kim et al. 2001, 2003). GAS is known to possess certain protective properties against cerebral ischemia injury through its anti-inflammatory and anti-oxidation effects (Peng et al. 2015). Relevant to this is activation of STAT3 in various physiological processes, such as cell proliferation, survival, and differentiation. Very interestingly, hypoxic or hypoxic-ischemic preconditioning has been shown to involve STAT3 signal pathway that that regulates inflammatory reaction. It has been reported that IL-1β can promote the proliferation, migration, and invasion of other cells via activating STAT3 (Fathima Hurmath et al. 2014); moreover, IL-1β acts through triggering the NF-κB pathway activation (Lee and Heur 2013).

While GAS is commonly known for its anti-inflammatory property, its regulatory effects on NLRP3 and NLRC4 inflammasome as well as STAT3 and NF-κB in astrocytes in cerebral ischemic injury have remained uncertain. This study sought to investigate the effects of GAS on NLRP3 and NLRC4 inflammasome in astrocytes in the adult rats subjected to middle cerebral artery occlusion (MCAO). In order to gain a fuller understanding of the anti-inflammatory mechanism of GAS, astrocytes in vitro were subjected to OGD exposure. The potential biochemical mechanism of GAS targeting specifically on the key inflammasome markers in OGD-stimulated astrocytes then followed. We report here that GAS can potentially improve the cerebral ischemic damage by inhibiting activation of NLRP3 and NLRC4 inflammasome coupled with suppression of STAT3 and NF-κB signaling pathways as evident also in OGD-treated astrocytes in vitro.

Materials and Methods

Animals, Source, and Maintenance

Male adult Sprague–Dawley rats ($n = 140$) weighing 285 ± 15 g were used in this study. Rats were provided by the Experimental Animal Center of Kunming Medical University, caged at room temperature 22 ± 2 °C, with access to food and water ad libitum. In the handling and care of animals, the guidelines as stipulated by the Opinions on Treating Experimental Animals of the Ministry of Science and Technology of China (2006) were followed. Rats were randomly divided into sham-operated group, middle cerebral artery occlusion (MCAO) group, and GAS + MCAO group.

In GAS + MCAO group, the rats were given an intraperitoneal injection of GAS 6 h prior to MCAO. In the MCAO group, the rats received an equal volume of normal saline instead of GAS In the sham-operated group, the carotid arteries were exposed but were neither ligated nor occluded.

At different time points after MCAO and corresponding sham control, rats were anesthetized via isoflurane. Following intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, the brain was removed and kept in the same fixative overnight containing 30% sucrose. Frozen coronal sections at 15 µm thickness were cut on a cryostat (Leica); after which they were processed for immunofluorescence staining as described below.

Middle Cerebral Artery Occlusion Surgical Procedure

The surgical procedure for MCAO followed that described previously by Longa (Longa et al. 1989). In brief, after anesthesia with isoflurane, the rats underwent a midline neck incision, and exposure of the common carotid artery (CCA) external carotid artery (ECA) and internal carotid artery (ICA), followed by separation and ligation of the CCA. After this, a nylon monofilament was inserted into the ICA distally from the ECA. The middle cerebral artery (MCA) was permanently occluded for 12 h, 1 day, and 3 days and sacrificed. In the sham operation group, only the blood vessels were separated without occluding the MCA. Daily intramuscular injection of penicillin was administered after the surgical procedure to prevent infection.

TNA2 Cells, Chemicals, and Reagents

TNA2 astrocytes were obtained from the ATCC in USA. Dulbecco's Modified Eagle's medium (DMEM) was purchased from Gibco. Fetal bovine serum (FBS) was obtained from Hyclone. Chemiluminescent horseradish peroxidase substrate and polyvinylidene fluoride (PVDF) membrane were purchased from Millipore. Antibodies directed against c-caspase-1, IL-18 were from Abcam. The antibodies against STAT3, phospho-STAT3, and NLRP3 were obtained from Cell Signaling Technology. The antibodies against NLRC4 were obtained from Abclonal. The antibody against NF-κB and actin, horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz Biotechnology. Unless otherwise stated, all other chemicals were purchased from Thermo. Gastrodin [GAS] (purity 98%) was purchased from Kunming Pharmaceutical Factory, Kunming, Yunnan, China.

Primary Astrocyte Cultures

All animal experimental procedures were carried out using protocols approved by the Experimental Animal Ethic Committee, China. Sprague–Dawley (SD) rats aged < 2 days were supplied by the Medical Experimental Animal Center of Kunming Medical University. Dissociation procedure of astrocytes of the postnatal rat brain followed that was described previously by Schaefer et al. (Bentz et al. 2006). Briefly, meninges-free cortices were cut into small cubes (1 mm^3) and digested with trypsin for 10 min at 37 °C. After this, the cell suspension was sieved through nylon filters. Cultures were maintained in DMEM with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. All cultures were incubated at 37 °C with 5%/95% CO_2/air (v/v) and 95% humidity. Cultures became confluent at about 2 weeks, which were used for this study.

TNA2 Cells and Treatments

TNA2 cell cultures were maintained in DMEM with 10% FBS and antibiotics (100 U/ml penicillin, and 100 mg/ml streptomycin) at a density not exceeding 5×10^5 cells/ml. All cultures were incubated at 37 °C with 5%/95% CO_2/air (v/v) and 95% humidity. On the day of the experiment, the culture medium (DMEM High Glucose) was removed. The cells were then rinsed with warm phosphate-buffered saline (PBS), followed by adding the experimental medium (DMEM Low glucose). Hypoxia preconditioning was performed when cells were cultured in a tri-gas incubator for 1, 4, 8, 16, and 24 h with 95% N_2 and 5% CO_2. Gastrodin at 10, 20, 50, and 100 µM was added 2 h before OGD stimulation. Stattic (20, 50, 100, and 200 µM), a specific STAT3 inhibitor, was used to determine if STAT3 pathway might be involved in the effect of GAS on TNA2 astrocytes.

Double Immunofluorescence Labeling

TNA2 and primary astrocytes derived from various treatments were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. After rinsing with PBS, the coverslips with adherent cells were used for double immunofluorescence labeling. Both brain sections mentioned above and TNA2/primary astrocytes were incubated overnight with goat anti-mouse GFAP (dilution 1:100), and anti-rabbit NLRC4 polyclonal antibody (dilution 1:300). Subsequently, the cells were incubated with TRITC-conjugated secondary antibody for 2 h at room temperature. Nuclear staining was by DAPI. The antibodies used in this study for Western blot and immunofluorescence are listed in Table 1. All images in the latter were captured with a fluorescence microscope (80; Nikon, Tokyo, Japan). The results are representative of at least three independent experiments.

Table 1 Details of antibodies used

Antibody	Company	Source	Dilution in WB	Dilution in immunofluorescence
β-actin	Santa Cruz	Mouse	1:2000	
Caspase-1	Abcam	Mouse	1:1000	
NF-κB	Santa Cruz	Mouse	1:2000	
p-STAT3	CST	Rabbit	1:2000	1:300
STAT3	CST	Rabbit	1:3000	
NLRP3	CST	Rabbit	1:1000	
NLRC4	Abclonal	Rabbit	1:1000	1:300
IL-18	Abcam	Rabbit	1:2000	
GFAP	Sigma	Mouse		1:100

Table 2 mRNA sequence

NLRP3	Sense: 5′-AGGGCTCTGTTCATTGGCT-3′ Antisense: 5′-AAATCTCTCGCAGTCCACCT-3′
NLRC4	Sense: 5′-TGGTCTTCTTCATCCGCCT-3′ Antisense: 5′-GGAGTCAGGTATGTTCACGAGC-3′
Caspase-1	Sense: 5′-GCTTCAGTCAGGTCCATCAG-3′ Antisense: 5′-CCCTTTCAGTGGTTGGCAT-3′
IL-18	Sense: 5′-CAACGAATCCCAGACCAGA-3 Antisense: 5′-CCTTCCATCCTTCACAGATAGG-3

Reverse Transcription-polymerase Chain Reactions (RT-PCR) (Table 2)

Total RNA was prepared from TNA2 by using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was reverse-transcribed using the Superscript TM-III kit (Invitrogen) with 3 mg total RNA. Images were captured with a Gel Doc 2000 image analyzer (Bio-Rad, Richmond, CA, USA). The results are representative of at least three independent experiments.

Western Blot Analysis

After various treatments and OGD exposure, the TNA2 astrocytes were washed with ice-cold PBS and lysed in ice-cold lysis buffer RIPA. Cell lysates were centrifuged at 12000 rpm for 30 min at 4 °C and the supernatants were then collected. Protein content was determined by using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (50 μg) were loaded per lane onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto immunoblot PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 2 h at room temperature and incubated separately with goat anti-rabbit

antibodies for STAT3, phospho-STAT3, NLRP3, NLRC4, IL-18, caspase-1, NF-κB, and β-actin antibodies overnight at 4 °C. The membranes were then washed three times for 15 min with TBS-T, and incubated with a 1:2000 dilution of horseradish peroxidase-coupled secondary antibodies for 2 h at room temperature. Blots were again washed three times for 5 min each in TBS-T and developed by the ECL detection system.

Statistical Analysis

Statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test, using SPSS (SPSS Inc, Chicago, IL, USA). The data were summarized and shown as mean ± SEM (standard error of mean) obtained from three independent experiments. Values of $p < 0.05$ were considered significant. Quantification of fluorescence intensity of immunofluorescence images was carried out using Grayscale analysis by Image J software.

Results

GAS Reduced NLRP3 and NLRC4 Inflammasome Protein Expression in Infarcted Cerebral Tissue Induced by MCAO

To determine the neuroprotective effect of GAS, rats were given GAS pretreatment followed by occlusion of the middle cerebral artery. The expression levels of NLRP3 and NLRC4 protein in the cerebral ischemic tissue were assessed at 12 h, 1 day, and 3 days after MCAO. Both NLRP3 and NLRC4 expression levels were significantly upregulated after MCAO between 12 h and 3 days compared with control. The increased expression of both proteins was attenuated by GAS. GAS significantly suppressed NLRP3 and NLRC4 protein expression levels after MCAO at 12 h and 3 days (Fig. 1a). Next, to gain further insights into activation of these cytokines, expression of downstream inflammatory proteins involved was assessed. GAS significantly decreased IL-18 and caspase-1 at protein level after MCAO at 12 h and 3 days (Fig. 1b). We also conducted immunofluorescence labeling to examine the level of NLRC4 in astrocytes identified by the specific marker GFAP. Immunofluorescence labeling showed that NLRC4 fluorescence intensity in GFAP labeled astrocytes was enhanced after MCAO at 1 day, but it was dampened when incubated with GAS (Fig. 1c). Finally, NLRC4 fluorescence intensity was statistically analyzed and plotted as bar chart Grayscale analysis by Image J software (Fig. 1d).
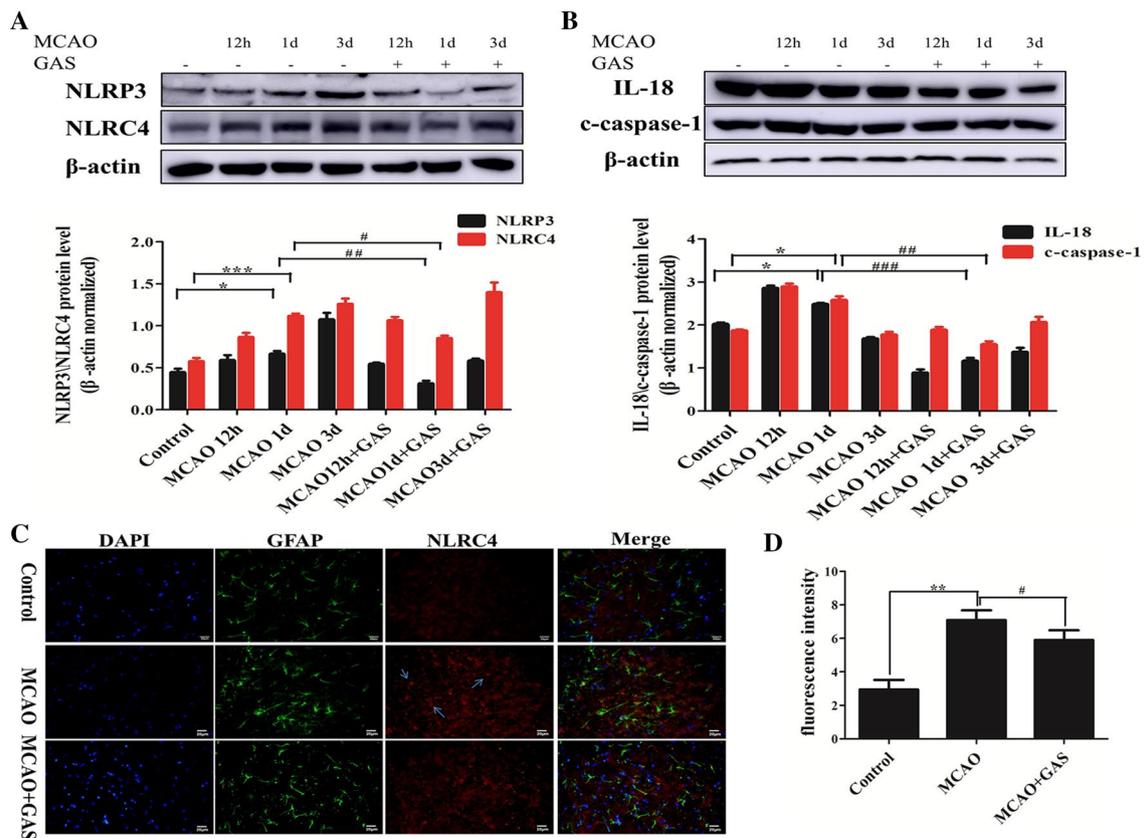
Fig. 1 Western blot showing Gastrodin reduced expression of NLRP3 and NLRC4 inflammasome protein levels induced by MCAO at 1 day (**a**). Western blot showing Gastrodin reduced IL-18 and caspase-1 protein expression levels induced by MCAO at 1 day (**b**). Immunofluorescence showing Gastrodin reduced expression of NLRC4 inflammasome (red) in astrocytes (GFAP$^+$ green) induced by MCAO at 1 day (**c**). Statistical analysis for Immunofluorescence pictures (**d**). Data are expressed as Mean ± S.D. and includes determination made in seven (n = 7) different animals from each group.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, versus control rats. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, versus MCAO-induced rats

GAS Decreased Expression of STAT3 Pathway Induced by Cerebral Ischemia

MCAO elicited increased phosphorylation of STAT3; however, rats pretreated with GAS followed by MCAO attenuated the effects i.e., pretreatment with GAS decreased the phosphorylation of STAT3 compared with the MCAO group. By immunofluorescence and Western blot, the phosphorylation level of STAT3 was decreased. As shown in Fig. 2, in the MCAO group, the immunofluorescence intensity of p-STAT3 was significantly increased in comparison with the control group. However, p-STAT3 intensity was decreased in the GAS-treated group (Fig. 2a, c). Finally, p-STAT3 intensity was statistically analyzed and plotted as bar chart (Fig. 2c).

OGD Induced Activation of NLRP3 and NLRC4 Inflammasome Proteins in TNA2 Astrocytes

In order to induce NLRP3 and NLRC4 activation in TNA2 astrocytes, the cells were cultured in a humidified airtight chamber equipped with an air lock and continuously flushed with 95%N_2/5%CO_2 for 1, 4, 8, 16, and 24 h. The protein expression levels of NLRP3 and NLRC4 were evaluated by Western blot which showed that expression levels of both proteins were substantially increased in OGD group when compared with the control group (OGD at 0 h), (Fig. 3a). Concomitantly, the protein expression levels of IL-18 and caspase-1, representing the downstream markers of inflammasome, were increased significantly when compared with the control (Fig. 3b).

Fig. 2 Western blot shows Gastrodin suppressed expression of p-STAT3 protein levels induced by MCAO at 1 day (**a**). Immunofluorescence shows Gastrodin attenuated p-STAT3 expression (red) in astrocytes GFAP + (green) induced by MCAO at 1 day (**b**). Statistical analysis for Immunofluorescence pictures (**c**). Data are expressed as Mean ± S.D. and includes determination made in seven ($n=7$) different animals from each group.*$p<0.05$, **$p<0.01$, versus SD wild-type rats. #$p<0.05$, ##$p<0.01$, versus MCAO-induced rats
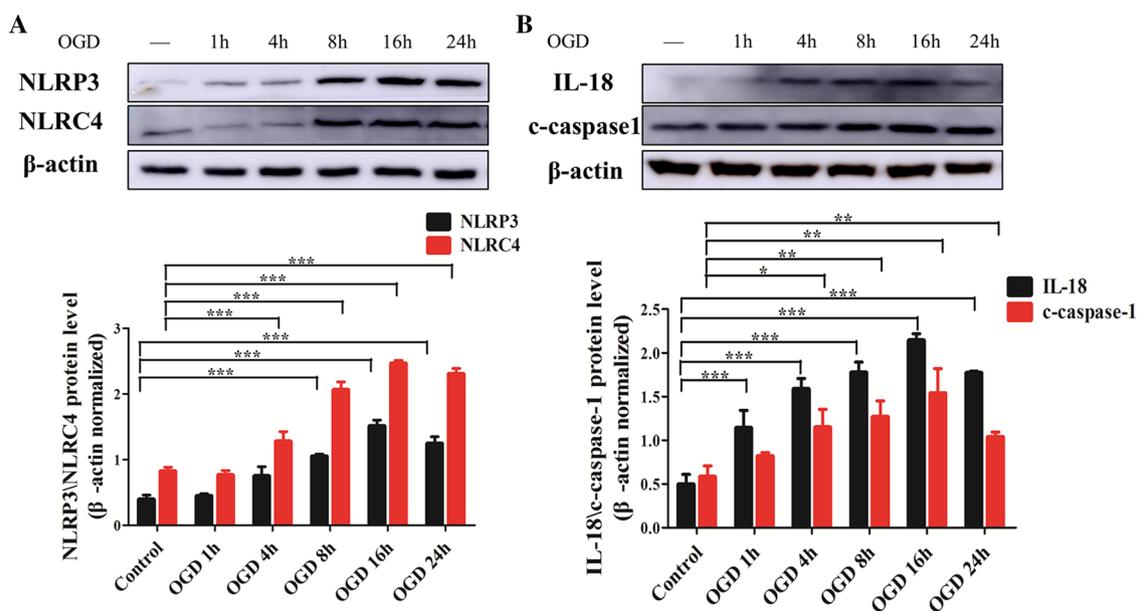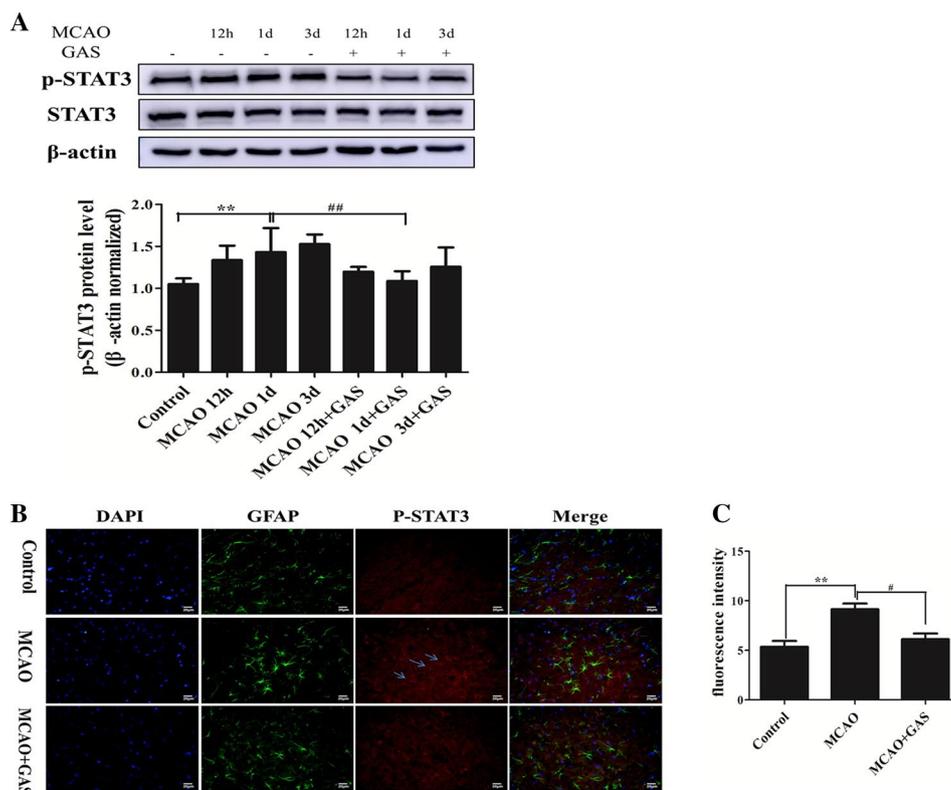

Fig. 3 Western blot shows OGD induced activation of NLRP3 and NLRC4 inflammasome in TNA2 astrocytes at different time points 4 h, 8 h, 16 h, and 24 h (**a**). Western blot showed OGD induced activation of IL-18 and caspase-1 in TNA2 astrocytes at different time points 1 h, 4 h, 8 h, 16 h, and 24 h (**b**). Data are expressed as Mean ± S.D. and includes determination made in six ($n=6$) different group.*$p<0.05$, **$p<0.01$, ***$p<0.001$, versus control TNA2 astrocytes

Gastrodin Reduced OGD-Stimulated Expression of NLRP3 and NLRC4 Inflammasome Proteins and mRNA in TNA2 Astrocytes

GAS is endowed with certain protective properties through its anti-inflammatory action. In view of this, we next investigated whether GAS would attenuate inflammasome activation. The cells were treated with different doses of GAS (10, 20, and 50 μM) for 2 h before OGD 16-h stimulation. GAS significantly suppressed NLRP3 and NLRC4 protein expression levels (GAS: 20 μM) and mRNA levels (GAS: 20 μM) in comparison with OGD-treated cells for 16 h (Fig. 4a, c). Additionally, GAS significantly decreased OGD-induced IL-18 and caspase-1 both at protein and mRNA expression levels (Fig. 4b). These findings support the notion that GAS can suppress inflammasome activation in OGD-stimulated TNA2 astrocytes.

Gastrodin Decreased OGD-stimulated Expression of NLRP3 and NLRC4 Inflammasome via STAT3 Signaling Pathway in TNA2 Astrocytes

STAT pathway is crucial for regulating the inflammatory responses in cerebral ischemia (Bao et al. 2013). We next determined whether GAS exerted its anti-inflammatory effect via regulating the STAT3 signal pathway in TNA2 astrocytes in OGD conditions. Under OGD conditions, p-STAT3 protein expression levels in astrocytes were markedly increased (Fig. 5a). GAS significantly inhibited the p-STAT3 protein expression in TNA2 astrocytes. We next treated the TNA2 astrocytes with different doses of Stattic (20, 50, 100, and 200 μM), an inhibitor of STAT3 for 1 h before OGD stimulation for 16 h. As in GAS-treated astrocytes stimulated with OGD, Stattic significantly inhibited expression of NLRP3 and NLRC4 as well as that of caspase-1 (Fig. 5b). Immunofluorescence
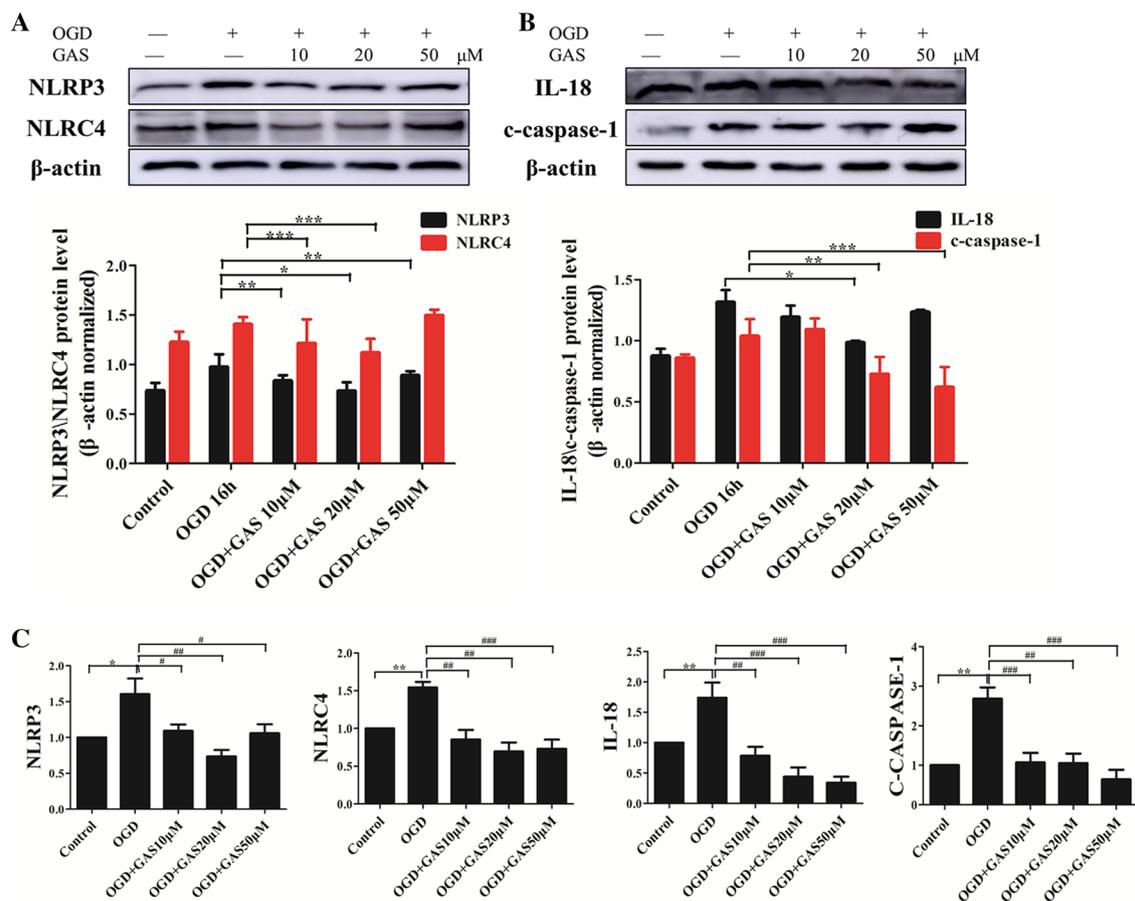

Fig. 4 Western blot showing Gastrodin at 20 μM reduced OGD-stimulated expression of NLRP3 and NLRC4 inflammasome protein levels in TNA2 astrocytes (**a**). Western blot showing Gastrodin at 20 μM reduced OGD-stimulated expression of IL-18 and caspase-1 inflammasome protein levels in TNA2 astrocytes (**b**). RT-PCR showing Gastrodin at 20 μM reduced OGD-stimulated expression of NLRP3, NLRC4, IL18, and caspase-1 inflammasome protein levels in TNA2 astrocytes (**c**). Data are expressed as Mean ± S.D. and includes determination made in five (*n* = 5) different groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, versus OGD 16-h-induced TNA2 astrocytes or control TNA2 astrocytes. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, versus OGD 16-h-induced TNA2 astrocytes

Fig. 5 Western blot showing p-STAT3 protein expression level was progressively increased with time in OGD (**a**). Western blot showing Stattic inhibited OGD-stimulated expression of NLRP3, NLRC4, and c-caspase-1 in TNA2 astrocytes (**b**). Statistical analysis for Immunofluorescence pictures (**c**). Data are expressed as Mean ± S.D. and includes determination made in six ($n = 6$) different groups.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, versus control TNA2 astrocytes or control primary astrocytes or OGD 16-h-induced TNA2 astrocytes. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, versus OGD 16-h-induced TNA2 astrocytes or OGD 16-h-induced primary astrocytes
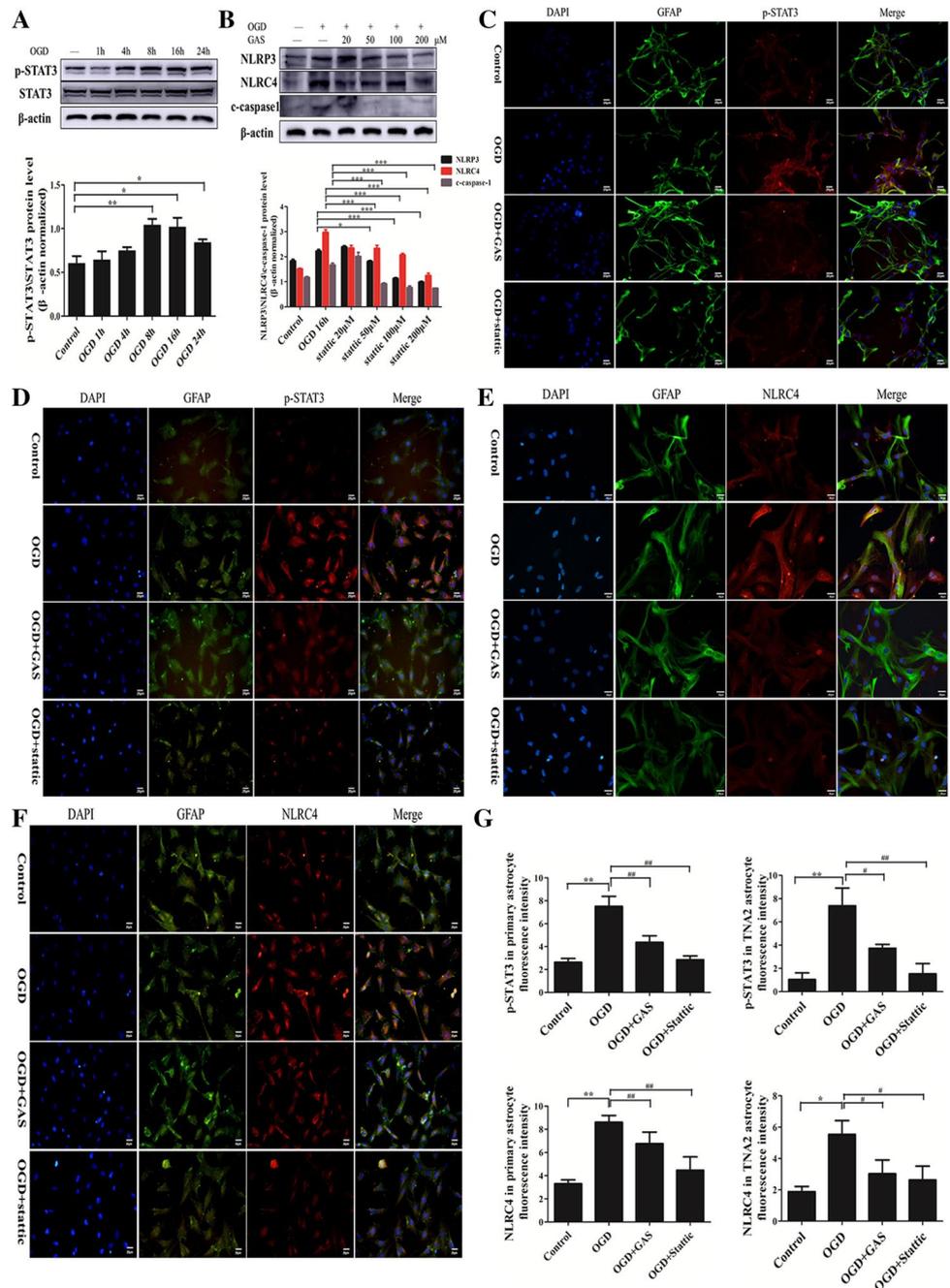
labeling showed that p-STAT3 and NLRC4 fluorescence intensity were also noticeably enhanced with OGD treatment but was dampened when incubated with GAS in primary astrocyte and TNA2 astrocyte (Fig. 5c–f). STAT3 and NLRC4 intensity were statistically analyzed and plotted as bar chart (Fig. 5g). The results indicate that STAT3 is essential for GAS-mediated inhibition of expression levels of inflammasome in TNA2 astrocytes.

Gastrodin Suppressed OGD-induced NF-κB Activation by Inhibition of STAT3 Signaling in Astrocytes

We next determined whether the inhibition of NF-κB activation by GAS and Stattic was mediated via STAT3 pathway. This is because activation of NF-κB pathway is thought to be a key signaling event involved in the activation of NLRP3 and NLRC4 inflammasome (Goh et al. 2009). Moreover, GAS has been reported to inhibit activation of the NF-κB

signaling (Yang et al. 2013). We showed here that indeed STAT3 inhibitor Stattic pretreatment inhibited OGD-stimulated NF-κB in TNA2 astrocytes (Fig. 6a). A similar inhibitory effect on NF-κB was observed in the GAS group, Stattic group, GAS + Stattic group (Fig. 6b). More importantly, the inhibitory effect of Stattic on NF-κB expression in OGD-stimulated astrocytes was paralleled by its inhibition of STAT3. Thus, the results indicated that inhibition of STAT3 is essential for GAS-mediated inhibition of NF-κB signaling pathway in astrocytes exposed to OGD.

Discussion

The present results have shown that MCAO and OGD induced a drastic increase in the expression of NLRP3 and NLRC4 inflammasomes along with increased expression levels of IL-18 and caspase-1 in astrocytes. Additionally, expression of p-STAT3 was increased in astrocytes in both experimental models. Remarkably, pretreatment with GAS inhibited or attenuated the effects of MCAO and OGD. In light of this, it is suggested that GAS can decrease OGD- and MCAO-stimulated expression of NLRP3 and NLRC4

inflammasome via STAT3 signaling pathway in astrocytes. It has been reported that NF-κB pathway plays a key role in the activation of NLRP3 and NLRC4 inflammasome. Here, we have shown that GAS also suppressed OGD-induced NF-κB activation by inhibition of STAT3 signaling in astrocytes as evident through use of its inhibitor Stattic. In brief, these findings have helped us to better understand the underlying mechanisms of the protective effects of GAS in ischemic conditions. Furthermore, the present result also supports the notion that GAS may serve as potential drug for treatment of ischemic brain injury.

Brain injury and its underlying molecular and biochemical mechanisms have been the subject of many studies in recent years focusing especially on the complicated and multiple signaling pathways in glial cells involved in the pathological process namely, the microglia and astrocytes. In cerebral ischemia, the local inflammation that is triggered can exacerbate the brain damage and promote the recurrence, which would influence both the prognosis and ultimate survival of patients (Faustino et al. 2011). It is well documented that inflammasome is a multiple protein complex that acts as a sensor and that it can detect host-derived danger signals and infectious agents (Li et al. 2013). There is ample
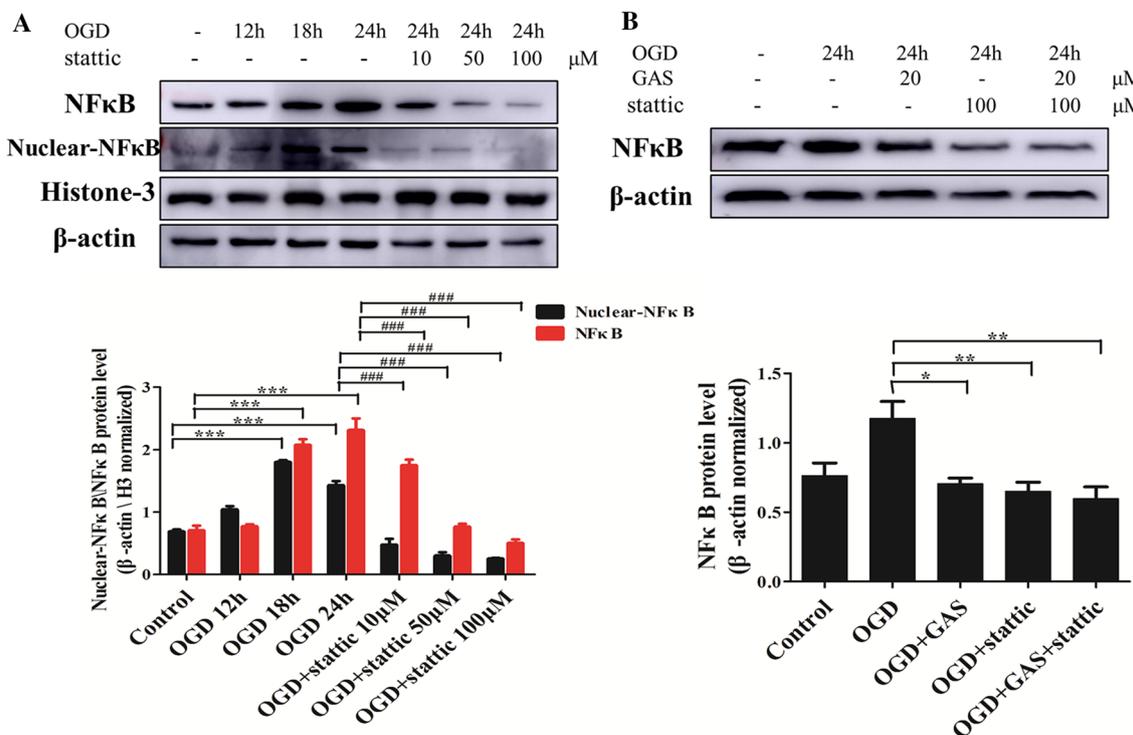

Fig. 6 Western blot showed suppression OGD-induced NF-κB activation by inhibition of STAT3 signaling with Stattic in TNA2 astrocytes (a). Western blot shows that GAS group, Stattic group, GAS + Stattic group exerted comparable inhibitory effect on NF-κB expression with the last-mentioned group being more pronounced (b). Data are expressed as Mean ± S.D. and includes determination made in seven or five ($n = 7$ or 5) different groups. $*p < 0.05$, $***p < 0.001$, versus control TNA2 astrocytes or OGD 24-h-induced TNA2 astrocytes. $^{###}p < 0.001$, versus OGD 24-h-induced TNA2 astrocytes

evidence demonstrating that inflammasome can exert a critical role in mediating inflammation in different diseases and among them maybe mentioned are cancer, ischemic brain injuries, neurodegenerative diseases, metabolic, and autoimmune disorders (Yang et al. 2014). The NLRP3 inflammasome is a macromolecular complex formed by NLRP3 linked to caspase-1 and is the focus of many studies. It is known to respond to PAMP or DAMP and induce the release of caspase-1-dependent pyroptosis (He et al. 2016). Procaspase-1 clustering leads to its auto-cleavage and formation of the active caspase-1 which induces the secretion of IL-1β and IL-18 proinflammatory cytokines (Schroder and Tschopp 2010). As opposed to NLRP3 which is induced by traditional conditions such as ischemia and hypoxia, NLRC4 is primarily studied in the context of microbial infection. It is interesting therefore to note that NLRC4 mRNA has been detected in the adult mouse brain (Poyet et al. 2001). Furthermore, NLRC4 mRNA was also detected in the brain tissue of Alzheimer's disease patients and that inflammasome activation was observed in astrocyte cultures (Liu and Chan 2014). Moreover, increased expression of NLRC4 was also associated with the inflammatory diseases such as Kawasaki disease (Ikeda et al. 2010) and atopic dermatitis (Macaluso et al. 2007). These authors have demonstrated increased gene expression of NLRC4 in patients when compared with the healthy controls thus emphasizing a role for NLRC4 in these inflammatory diseases. Despite these findings, the specific mechanism of NLRC4 in the above-mentioned diseases has not been fully elucidated. It is well documented that astrocytes constitute a major component of glial population in the CNS (Ling and Leblond 1973), and they are known to partake in various essential functions in the brain including buffering the extracellular space, providing nutrition, support, and protection to neurons, interchanging glutamate and glutamine for synaptic transmission with neurons, and play crucial roles in the CNS, etc. As one of the key players mediating inflammatory response, reactive astrocytes that are featured prominently in different neurodegenerative diseases and neurological disorders are known to produce pro- and anti-inflammatory cytokines, trophic factors, and chemokines (Lau and Yu 2001) which are crucial to neuronal survival, maturation, and neurogenesis (Emsley et al. 2004). As the role of the inflammasome in astrocytes remains obscure, we have used the MCAO in vivo and the OGD in vitro mimicking the cerebral ischemia to decipher its involvement in astrocytes in altered conditions. We have shown that the expression levels of NLRP3 and NLRC4 inflammasomes as well as that of IL-18 and caspase-1 were markedly increased in astrocytes both in MCAO and OGD.

Previous study had reported that GAS pretreatment could decrease the glutamate/GABA ratio during ischemia (Zeng et al. 2007). Moreover, GAS markedly decrease the infarct and edema volume in MCAO rats at 24 h (Zeng et al. 2006)

thus indicating its neuroprotective capability. In our earlier study, we had also shown that GAS inhibited LPS-induced expression of proinflammatory mediators and cytokines in the mouse BV-2 microglia cells (Dai et al. 2011), but the underlying mechanism of GAS in neuroprotection had remained dubious. Here, we have provided experimental evidence that GAS down-regulated MCAO and OGD-induced release of inflammasome NLRP3 and NLRC4 and proinflammatory cytokines IL-18 in MCAO brain, more specifically in astrocytes, pretreated with GAS. It is unequivocal from the present results that when treated with GAS, the expression levels of NLRP3, NLRC4, caspase-1, and IL-18 in MCAO and OGD were significantly suppressed. It remains uncertain whether Gastrodin can exert its suppression effects either directly or indirectly on reactive astrocytes in MCAO or TNA2 astrocytes. It has been reported that astrocytic reaction maybe mediated by activated microglia (Fang et al. 2016). Therefore, the possibility that GAS acts on reactive astrocytes through activated microglia should be considered.

Many cellular and molecular signaling pathways have been reported to be involved in the pathological changes in the aftermath of ischemic brain injury and among them may be mentioned is STATs which participate in many cellular activities (O'Shea et al. 2013). It has been reported that STAT3 is activated in different brain cells including neurons, astrocytes, microglia, endothelial cells, and macrophages during neuronal development, neuroprotection, and regeneration after ischemic injury (Yu et al. 2013). It is therefore relevant to note in the present results that the STAT3 pathway is activated in astrocytes in MCAO and OGD. Additionally, we have shown Stattic, a STAT3 inhibitor, significantly decreased the inflammasome NLRP3, NLRC4, caspase-1 and IL-18 in astrocytes. Taken together, the present results suggest that GAS can decrease OGD-stimulated expression of NLRP3 and NLRC4 inflammasome via STAT3 signaling pathway in TNA2 astrocytes.

Another signaling to be considered is NF-κB that is thought to be a key signaling pathway involved in the activation of NLRP3 and NLRC4 inflammasome (Goh et al. 2009). NF-κB plays a pivotal role in the transcriptional control of different inflammatory mediators, including TNF-α, IL-6, pro-IL-1β, pro-IL-18, procaspase-1, and NLRP3 (Choi and Ryter 2014). Its activation is not only required for the assembly of inflammasome complex but is also required for the activation and recruitment of inflammatory cells to the site of inflammation. In view of its wide contribution in inflammatory response, it is desirable to identify an appropriate or effective NF-κB inhibitor to mitigate the neuroinflammatory damage induced by cerebral ischemia. In this regard, we have previously reported that GAS could effectively inhibit activation of the NF-κB signaling (Yang et al. 2013). Therefore, Gastrodin and Stattic, either separately or

in combination, could inhibit the NF-κB pathway in reactive astrocytes. In other words, GAS can exert its effects on different pathways including STAT3 and NF-κB signaling pathways among others in reactive astrocytes in cerebral ischemia.

Conclusions and Future Perspectives

This study has provided the first morphological and biochemical evidence demonstrating that GAS can reduce MCAO- and OGD-stimulated expression of inflammasome in the astrocytes. We have further shown that inhibition of STAT3 and NF-κB is a putative mechanism via which GAS can inhibit activation of inflammasome and proinflammatory cytokines production in response to MCAO and OGD in astrocytes. The present results suggest that both STAT3 and NF-κB are two possible signaling pathways by which GAS can exert its neuroprotection effects. Nonetheless, arising from the present results, the underlying mechanism of GAS in exerting its neuroprotective effects is now better clarified. Thus, GAS may hold promises as a therapeutic agent for amelioration of neuroinflammation mediated by reactive astrocytes in cerebral ischemia.

Compliance with Ethical Standards

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

Ethical Approval of Animal Studies All animal procedures and studies were carried out in accordance with the guidelines as stipulated by the Opinions on Treating Experimental Animals of the Ministry of Science and Technology of China (2006).

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