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Gastrodin Regulates PI3K/AKT-Sirt3 Signaling Pathway and Proinflammatory Mediators in Activated Microglia

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Abstract

Activated microglia and their mediated inflammatory responses play an important role in the pathogenesis of hypoxic-ischemic brain damage (HIBD). Therefore, regulating microglia activation is considered a potential therapeutic strategy. The neuroprotective effects of gastrodin were evaluated in HIBD model mice, and in oxygen glucose deprivation (OGD)-treated and lipopolysaccharide (LPS)activated BV-2 microglia cells. The potential molecular mechanism was investigated using western blotting, immunofluorescence labeling, quantitative realtime reverse transcriptase polymerase chain reaction, and flow cytometry. Herein, we found that PI3K/AKT signaling can regulate Sirt3 in activated microglia, but not reciprocally. And gastrodin exerts anti-inflammatory and antiapoptotic effects through the PI3K/AKT-Sirt3 signaling pathway. In addition, gastrodin could promote FOXO3a phosphorylation, and inhibit ROS production in LPSactivated BV-2 microglia. Moreover, the level P-FOXO3a decreased significantly in Sirt3-siRNA group. However, there was no significant change after gastrodin and siRNA combination treatment. Notably, gastrodin might also affect the production of ROS in activated microglia by regulating the level of P-FOXO3a via Sirt3. Together, this study highlighted the neuroprotective role of PI3K/AKT-Sirt3 axis in HIBD, and the anti-inflammatory, anti-apoptotic, and anti-oxidative stress effects of gastrodin on HIBD.

Keywords Gastrodin · Microglia · Hypoxic-ischemic brain damage · PI3K/AKT–Sirt3

Introduction

Hypoxic-ischemic brain damage (HIBD) in newborns is caused by perinatal asphyxia, leading to brain injury and high mortality rates [1–5]. Surviving infants often suffer from lifelong neurological issues like cerebral palsy and intellectual disability, significantly impacting their quality of life [6, 7]. However, the pathogenesis of perinatal HIBD has not been fully determined.

Increasing evidence suggests that inflammation plays an important role in neonatal hypoxic-ischemic brain injury [8]. Microglia, which are resident immune cells in the brain,

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play a crucial role in innate immune responses [9–12]. They are activated early in cerebral ischemia and trigger the release of local pro-inflammatory cytokines [13–15]. Microglia continuously monitor changes in brain homeostasis and respond to specific signaling molecules expressed on, or released by, surrounding cells [10]. Previous studies have shown that microglia can be classified into "M1" (pro-inflammatory) and "M2" (anti-inflammatory) states following pathogenic stimuli, in which the M1 subtype expresses CD16/32, TNF-α and the M2 subtype expresses CD206, Arg-1, TGF-β1, and other biomarkers [16–20]. In the developing hypoxic cerebellum, activated microglia cause neuronal damage by producing interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [21]. Furthermore, the activation and aggregation of microglia in the dentate gyrus have been reported as markers of mild hypoxic-ischemic brain injury in neonates [22]. Therefore, identifying the activation of microglia and their mediated inflammatory response after hypoxicischemic brain injury is a crucial step in the treatment of HIBD.

Currently, hypothermia therapy is the recommended intervention for the treatment of neonatal hypoxic-ischemic brain injury [23]. However, because of its limited efficacy [23–25], there is an urgent need to find safer and more effective complementary therapies. *Gastrodia elata*, a plant in the orchid family, which has been cultivated for thousands of years in China, was observed to have anticonvulsive and neuroprotective effects [26]. Gastrodin (C13H18O7) is the active ingredient of *Gastrodia elata* and is considered a potential medicine. Studies have shown that gastrodin can inhibit the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated microglia [27]. In our previous study [1], we found that gastrodin could significantly inhibit the expression of Notch signaling pathway and increase sirtuin 3 (Sirt3) expression in LPS-induced BV-2 microglia after HIBD, thus playing a neuroprotective role. Furthermore, gastrodin has been shown to alleviate cerebral ischemia injury in mice by enhancing antioxidant and anti-inflammatory activities and inhibiting apoptosis pathways [28].

The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) pathway is critical for cell growth, metabolism, inflammation, cell survival, and other signaling pathways [29]. PI3Ks are intracellular lipid kinases involved in the regulation of cell proliferation, differentiation, and survival [30]. Studies have suggested that activation of the PI3K/AKT signaling pathway plays an important role in ischemic brain injury [31]. Moreover, this pathway is also involved in the survival and death mechanism of nerve cells [32]. Activation of the PI3K/AKT pathway increases the phosphorylated (P)-AKT level, inhibits the expression of nuclear factor kappa B (NFκB), promotes the expression of B-cell CLL/lymphoma 2 (Bcl-2), and reduces the inflammatory response and apoptosis after HIBD [29]. Notably, the PI3K/AKT pathway also plays a crucial role in the activation of microglia. In Alzheimer's disease (AD), regulation of the PI3K/AKT/glycogen synthase kinase 3 beta (GSK-3β) pathway has a neuroprotective effect on LPS-induced neuroinflammation in BV-2 cells [33]. Moreover, the PI3K/AKT pathway is associated with the expression of phenotypic markers of M1 and M2 microglia in LPS-activated BV-2 cells [34]. Additionally, reactive oxygen species (ROS) can promote microglia apoptosis by affecting the PI3K/AKT signaling pathway in oxygen-glucose deprivation (OGD) induced microglia after cerebral ischemia-reperfusion injury [35]. Therefore, it is important to further explore the role of the PI3K/AKT signaling pathway in activated microglia after HIBD.

Sirtuins are protein deacetylases that depend on niacinamide adenine dinucleotide (NAD) [36]. As a typical member of mitochondrial Sirtuin family, Sirt3 can combat oxidative stress, resist cell apoptosis, prevent cell aging and tumors [37–40], and plays a role in regulating and maintaining basic ATP levels [41]. However, Sirt3 appears to be associated with the PI3K/AKT pathway. Studies have shown that C-X-C motif chemokine ligand 6 (CXCL6) regulates Sirt3 expression by activating AKT/forkhead box O3a (FOXO3a), thereby regulating cell permeability, proliferation, and apoptosis after ischemia-reperfusion injury [42]. Downregulating Sirt3 or inhibiting the activity of thePI3K/AKT/FOXO3a pathway also weakened the protective effect on oxidative stress and apoptosis induced by high glucose [43]. Consequently, the aim of this study was to explore whether gastrodin affects the function of activated microglia by regulating the PI3K/AKT signaling pathway and Sirt3 expression in neonatal HIBD mice and endotoxin- and oxygen-glucose-deprived BV-2 microglia. We also sought to determine the underlying mechanism by which gastrodin acts on activated microglia via the PI3K/AKT-Sirt3 pathway.

Materials and Methods

HIBD Animal Model

This study was conducted within the appropriate ethical framework. All experimental protocols were approved by the Animal Research Ethics Committee of Kunming Medical University (kmmu20211454). The C57BL/6J mice were provided by the Experimental Animal Center of Kunming Medical University. In this study, we used c57 mice at 9–11 days after birth, which is close to that of 37 weeks old

human fetuses [44]. Mice were housed at 22 ± 2 °C under a 12 h light/dark cycle with *ad libitum* access to water and food. The mice were randomly divided into a sham group, an HIBD group, and an HIBD + gastrodin pretreated group (HIBD + G). The mice were anesthetized with isoflurane, and the left common carotid artery was exposed and ligated. Subsequently, the mice were placed in anoxic chamber containing 8% oxygen and 92% nitrogen at 28–30 ℃ for 40 min as reported by Min et al. [45]. The sham group received the same surgical procedure, but without ligation of the common carotid artery. In the HIBD + G group, mice were intraperitoneally injected with gastrodin (dissolved in normal saline) at a dose of 100 mg/kg three times: 1 h before carotid artery ligation, immediately after, and 12 h after hypoxia [46]. Gastrodin (purity 99.7%) was supplied by Kunming Pharmaceutical Co., LTD. (Kunming, China). The mice in each group were anesthetized using isoflurane and sacrificed at various time points (1 day and 3 days) after HIBD, depending on the purpose of the experiment. We carrried out 2,3,5-Triphenyltetrazolium chloride (TTC) staining, Nissl staining, Tunel staining, immunofluorescence staining, and tissue protein extraction. In addition, the mice at various time points (1, 3, and 7 days) after HIBD were evaluated for their weight.

Neurological Tests

The Zea-Longa scores were defined based on the following criteria: 0 indicating no neurologic deficit; 1 indicating failure to extend contralateral forepaw fully; 2 indicating circling to the outside; 3 indicating falling to the right; and 4 indicaating unable to walk spontaneously and having a depressed level of consciousness [47].

Grip test: mice were suspended by both their forepaws on a fine wire rod (diameter, 1.5 mm) stretched horizontally 50 cm over a cotton pad, Time before falling was recorded [48]. Geotaxis test:the mice were placed head downward on a 45° inclined board, and the time taken for the mice to turn to head upward was recorded [49]. All behavioural experiments were observed jointly by two uninformed laboratory personnel and were carried out in strict accordance with the scoring rules.

Nissl Staining

After paraffin sections were dewaxed, they were soaked in cresol violet staining solution, washed quickly with distilled water, and then placed in Nissl differentiation solution. Finally, the slices were cleared in pure xylene and then coated with a neutral gel for use in an optical microscope with magnifications.

TUNEL Staining

TUNEL test kit (G1501; Servicebio) examined fragments of DNA in paraffin sections 7 μm thick to assess cell death. The nucleus was stained with DAPI. Finally, the stained sections were observed under a fluorescence microscope.

BV-2 Microglia Cell Viability Assay

To determine the cytotoxic effect of gastrodin on BV-2 microglia, the cells were respectively plated into 96-well microplates and cultured for 24 h. The cells in each well were treated with gastrodin (0–1 mg/mL) for 1 h. Then, the cells were removed and incubated in Cell Counting Kit-8 (CCK-8) solution under dark conditions (CCK-8 solution:medium = 1:100). They were then incubated in an incubator at 37℃ for 3 h. Finally, the corresponding absorbance (optical density) value of the cells was read at 450 nm wavelength using a microplate analyzer.

ELISA Assay

The cell supernatant was collected after LPS treatment and determined by ELISA. The protein levels of TNF-a were detected with the kit (EMC102a) of Xinbosheng Biotechnology Co., LTD.

BV-2 Cell Culture and Treatment with LY294002

BV-2 microglia were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37 °C. Cells were inoculated into six-well plates at a density of 6×10^5 cells /mL per well. Subsequently, BV-2 microglia were treated with different substances according to the experimental requirements: LPS (1 μg/mL, Sigma, St. Louis, MO, USA) stimulation to establish an inflammation model, and oxygen glucose deprivation (OGD) treatment to establish an HIBD model in vitro. The cells were randomly divided into four groups: Control (Con), LPS (LPS)/OGD, LPS + gastrodin (LPS + $G_{0.17\ mM}$)/ OGD + gastrodin (OGD + $G_{0.17\ mM}$), and LPS + gastrodin (LPS + $G_{0.34\ mM}$)/ OGD + gastrodin (OGD + $G_{0.34mM}$). Two different concentrations of gastrodin were used, based on cell viability assays determined in our previous study [46]. In addition, the cells were pretreated with LY294002 (15 μM, Selleck, Houston, TX, USA) before the other treatments, and divided into the following groups: control (Con), LY294002, LPS, LPS + LY294002, LPS + gastrodin (LPS + G) and LPS + LY294002 + gastrodin (LPS + LY294002 + G); Control (Con), OGD, OGD + LY294002, OGD + LY294002 + gastrodin (OGD + LY294002 + G). Before that, the cells were treated

with different concentrations of LY294002 (10 μM, 15 μM) to determine the appropriate concentration for use in this experimen. Under the same conditions, the cells were treated with 0.34 mM gastrodin. After that, immunofluorescence staining and cell protein extraction were performed for western blotting analysis.

Treatment of BV-2 Microglia with Sirt3-Short Interfering RNA (siRNA)

Transfection was performed using the Lipofectamine 3000 Transfection reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. BV-2 microglia cells were grouped as follows: Control group (Control), negative control group (siRNA-NC), *Sirt3*-siRNA group, *Sirt3*-siRNA + gastrodin group (siRNA + G), *Sirt3*-siRNA + LPS group (siRNA + LPS), and *Sirt3*-siRNA + LPS + gastrodin group (siRNA + LPS + G). Cells were inoculated in six-well plates at a density of 6×10^5 cells /ml. When the cell density reached 30–50%, the transfection complex was prepared in Opti-MEM Medium (Gibco, Grand Island, NY, USA) and siRNA (50 nM). BV-2 cells were then mixed with the transfection complex and incubated in an incubator for 48 h. The same procedure as described above was followed for gastrodin (0.34 mM) and LPS intervention. Cells were collected for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis. Three pre-designed *Sirt3*-siRNA sequences were used: Si-Sirt3-1 (sense: CCCUGAAGCCAUCUUUGAATT, antisense: UUCAAAGAUGGCUUCAGGGTT); Si-Sirt3-2 (sense: GCAAGGUUCCUACUCCAUATT, antisense: UAUGGAGUAGGAACCUUGCTT); and Sirt3-3 (sense: GGCUCUAUACACAGAACAUTT, antisense: AUGUUCUGUGUAUAGAGCCTT). Additionally, different concentrations of siRNA were transfected to determine the optimal concentration for use in the final experiment.

Immunofluorescence Labeling of Microglia in the Corpus Callosum of HIBD Mice and BV-2 Microglia

The mice were sacrificed, and their brains removed and embedded in paraffin wax. Coronal sections with a thickness of 4–5 μm were cut and subjected to antigen repair using citric acid buffer solution. The cells were fixed with 4% paraformaldehyde for 30 min. Cells or tissue sections adhered to slides were then used for immunofluorescence staining. After washing with phosphate buffered saline (PBS) several times, the tissue sections and cells were blocked with 5% goat serum at room temperature. The sections were then incubated overnight in a humidifier box at 4 °C with the following primary antibodies: anti-CD16/32 (1:200; Rabbit monoclonal; Abcam, Cambridge, MA, USA; ab223200), anti-CD206 (1:200; Rabbit polyclonal; Proteintech, Rosemont, IL, USA; 18704-1-AP), anti-ionized calcium-binding adapter molecule 1 (IBA1) (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-32,725), anti-PI3K (1:200; Rabbit polyclonal; Origene Technologies, Rockville, MD, USA; AP20772PU-N), anti-phosphorylated (P)-PI3K (1:200; Rabbit polyclonal; ORIGENE; TA325781), AKT (1:200; Mouse monoclonal; Origene Technologies; TA504230) and P-AKT (1:200; Rabbit polyclonal; ORIGENE; TA325218). After washing with PBS, the slides were incubated with Cy3-conjugated secondary antibody (1:200; Sigma; C2306), fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma; F0257) and FITC-conjugated lectin (*Lycopersicon esculentum*) (1:200; Sigma; L0401), respectively, at room temperature for 1 h. After washing with PBS, the sections were sealed with a fluorescent sealer containing 4', 6-diaminyl-2-phenylindole (DAPI). Images were obtained under a laser scanning confocal microscope (Nikon, Tokyo, Japan) and a fluorescence microscope (Axio Observer ZI (Zeiss, Oberkochen, Germany). The experiment was carried out three times and data analysis was performed using ImageJ (NIH, Bethesda, MD, USA).

Western Blotting of Proteins in the Corpus Callosum of HIBD Mice and BV-2 Microglia

The mice were sacrificed at 1d and 3d after HIBD, respectively. The corpus callosum of each group was extracted by fresh dissection, and then rapidly frozen in liquid nitrogen and stored at −80 °C. The corpus callosum was ultimately chosen for this study because it contains a large number of microglia in the brain of rats after birth [50]. The extracted tissue was treated with a protein extraction reagent containing a protease inhibitor. The protein concentration was measured using a protein assay kit. The BV-2 cells were washed twice with PBS and lysed in lysis buffer (1 × radioimmunoprecipitation assay (RIPA) lysis buffer and protease inhibitor mixture) for 10 min at 4 °C. The mixture was centrifuged at 14,000 x *g* and 4 °C for 20 min, and the supernatant was collected for further analysis. An equal amount of protein was separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% skim milk. The membrane was incubated overnight at 4 °C with the primary antibody (Table 1). Next day, the membrane was incubated with anti-mouse IgG or anti-rabbit IgG conjugated with horseradish peroxidase (HRP) for 1 h. After washing, the protein bands were detected by a chemiluminescence imaging instrument. All experiments were repeated at least three times and data analysis was performed using ImageJ.

Table 1 Antibodies used for immunostaining and Western blotting

Anti-body	Host	Source	Catalog number
CD16/32	Rabbit monoclonal	Abcam	ab223200
IBA-1	Mouse monoclonal	Santa Cruz	sc-32725
Cy3	Sheep	Sigma	C2306
FITC	Goat	Sigma	F0257
PI3K	Rabbit polyclonal	ORIGENE	AP20772PU-N
P-PI3K	Rabbit polyclonal	ORIGENE	TA325781
AKT	Mouse monoclonal	ORIGENE	TA504230
P-AKT	Rabbit polyclonal	ORIGENE	TA325218
TNF-α	Rabbit polyclonal	Millipore	AB1837P
TGF-β1	Rabbit polyclonal	ORIGENE	TA313319
Arg-1	Mouse monoclonal	Proteintech	66129-1-lg
Bax	Rabbit polyclonal	Affinity	AF0120
Bcl-2	Mouse monoclonal	Affinity	BF9103
β-actin	Mouse monoclonal	ORIGENE	TA811000
IL-10	Mouse monoclonal	Abcam	ab33471
CD206	Rabbit monoclonal	Proteintech	18704-1-AP
P-FOXO3a	Rabbit polyclonal	Affinity	AF3020

RNA Isolation and qRT-PCR

Total RNA was extracted from BV-2 microglia using Trizol (Ambion by Life Technologies, Foster City, CA, USA) and quantified by spectroscopy. cDNA was synthesized using a reverse transcription kit (Takara, Dalian, China; RR047A) according to manufacturer's instructions. The cDNA was then used as the template for the qPCR step of the qRT-PCR protocol. The reaction mixture (20 μL) was prepared according to the kit instructions (Takara, RR820A) as follows: 10 μL of TB Green Premix Ex Taq II, 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), 2 μL of cDNA template, 0.4 μL of ROX Reference Dye or Dye II, 6 μL of RNase-free ddH2O. The *Actb* gene (encoding β-actin) was used as an internal reference for normalization. The relative mRNA expression of the target genes was calculated using the $2^{-\triangle\triangle Ct}$ method [51], and the experiment was carried out three times independently. Primer sequences: *Akt* (designed according to the mouse sequence in the NCBI database) (sense: TCGTGTGGCAGGATGTGTATG, antisense: TAGGAGAACTTGATCAGGCGG); *Sirt3* [52] (sense: TTTCTTTCACAACCCCAAGC, antisense: AGGGATCCCAGATGCTCTCT); *β-actin* (sense: GTGGGAATGGGTCAGAAGGA, antisense: TACATGGCTGGGGTGTTGAA).

ROS Detection Using Flow Cytometry

BV-2 cells were plated in six-well plates at a density of 6×10^5 cells /ml. The different treatment groups included: Control, LPS (LPS), LPS + gastrodin (0.17 mM) (LPS + G0.17 mM), and LPS + gastrodin (0.34 mM) (LPS + G0.34 mM). The treatment with drugs was performed as described previously. The dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe (1:1000; Beyotime, Jiangsu, China) was added to the cells and incubated at 37 ℃ and 5% CO_2 for 1 h. The cells were then centrifuged, washed twice with PBS, and suspended in PBS. The cells were analyzed using FACSCelesta™ 3 flow cytometry (BD Biosciences, San Jose, CA, USA) under FITC fluorescence condition. The experiment was repeated three times, and the data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Statistical Analysis

SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. After the homogeneity test of variance, multiple comparisons were made using one-way analysis of variance (ANOVA) and Dunnet post-hoc tests were used to determine the statistical significance of the differences between the groups. All experiments were conducted in triplicate from different tissue or cell samples. $P < 0.05$ was considered statistically significant.

Results

Gastrodin Reduced the Cerebral Infarct Volume and Improved Growth and Neurological Deficits in Mice After HIBD

TTC staining was applied to detect the cerebral infarct size in mice after HIBD. The results showed that HIBD injury caused a large area of brain infarction compared with that in the sham group. After gastrodin treatment, the cerebral infarct volume was reduced (Fig. 1A, B).

The body weight of HIBD mice was decreased and their development was hindered when compared with the sham group. However, these were alleviated after gastrodin intervention (Fig. 1C). In addition, neurological tests of mice were evaluated at the 1st, 3rd, 7th day after operation. The Zea-Longa scores in HIBD group were higher than that in the Sham group at various time points. In the HIBD + GAS group, the scores were significantly lower than that in the HIBD group at 3 and 7 days; there was no obvious difference between the 1st day after operation (Fig. 1D). The Grip test showed that the muscle strength tolerance of mice was reduced in HIBD group compared to Sham group at every time point, and in HIBD + GAS group was significantly stronger than that in HIBD group at the 3rd, 7th day, while there was no obvious difference between the 1st day after operation (Fig. 1E). The HIBD mice exhibited prolonged Geotaxis reflex compared to the Sham group in 1st, 3rd day after operation, and the time of geotaxis reflex in

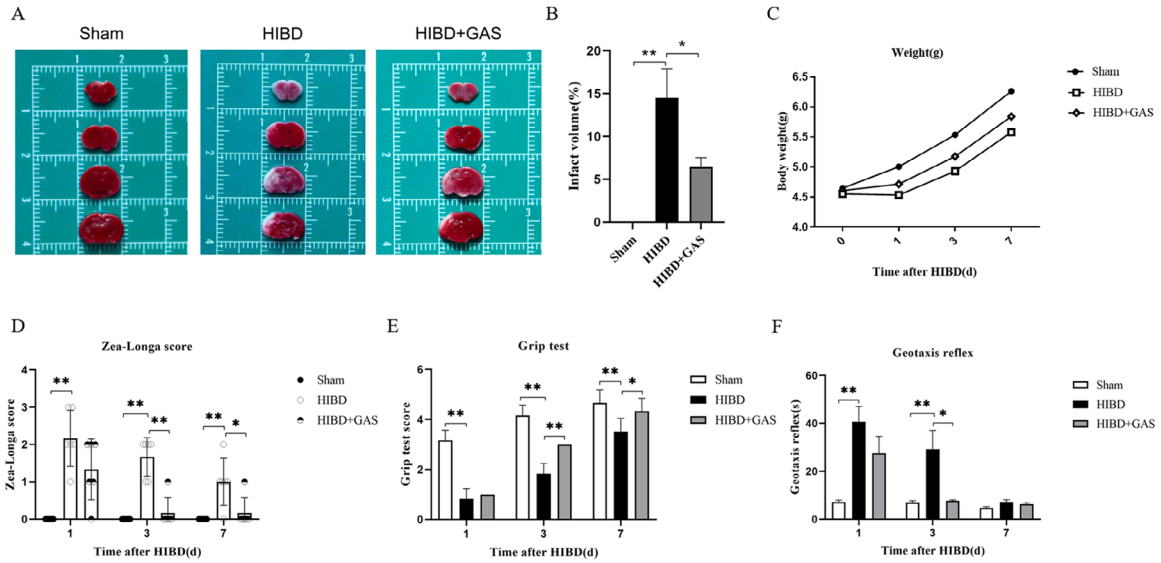

Fig. 1 Gastrodin reduced the cerebral infarct volume and ameliorated neurological dysfunction after HIBD. **(A)** Representative images of mouse brain tissue in each group after TTC staining. **(B)** Quantification of the cerebral infarct area in each group of mice. **(C-F)** Quantification of body weight, Zea-Longa score, grip test, and geotaxis reflex in each group. *P < 0.05; **P < 0.01; **P < 0.001. The values represent the mean ± SEM in triplicate

HIBD + GAS group was shortened in 3rd day, while there was no obvious difference at the 1st,7th day (Fig. 1F).

The results of Nissl staining and Tunel staining showed that compared with sham group, the number of necrotic and apoptotic neurons was significantly increased in the infarction and penumbral area at 1d and 3d after HIBD. Gastrodin was obviously found to decrease the incidence of cells undergoing apoptosis in the infarcted cerebral cortex and penumbral region in HIBD mice(Supplementary Figs. 1, 2). These findings suggest that gastrodin can effectively reduce neuronal damage and promote neurological recovery.

Gastrodin Decreased TNF-α and CD16/32 Levels and Apoptosis of Microglia, but Increased the Levels of TGF-β1, Arg-1, and *CD206 in the Corpus Callosum of HIBD Mice*

Western blotting analysis showed that the levels of TNF-α and Bcl-2 associated X protein (Bax)/Bcl-2 were upregulated in 1d and 3d HIBD mice, but they were downregulated after gastrodin treatment. However, transforming growth factor beta 1 (TGF-β1) and arginase 1 (Arg-1) levels were decreased in HIBD mice, and increased after gastrodin treatment(Fig. 2). Immunofluorescence labeling showed that CD16/32 levels were enhanced at the 1d and 3d HIBD mice, but were significantly decreased after gastrodin treatment (Fig. 3). However, CD206 levels were reduced at 3d HIBD mice, while the expression was significantly increased after gastrodin treatment at 1d and 3d after HIBD (Fig. 4).

Gastrodin Decreased the Levels of TNF-α and CD16/32 in OGD-Induced BV-2 Microglia, but Increased the Levels of TGF-β1 and Arg-1

CCK-8 assessment of the cytotoxicity of gastrodin on BV-2 microglia did not result in significant toxic effects (Supplementary materials Fig. 3A).Western blotting analysis showed that the levels of TGF-β1 and Arg-1 decreased significantly in OGD-induced BV-2 microglia, while the levels of TNF-α and CD16/32 increased significantly. Gastrodin increased TGF-β1 and Arg-1 levels, and significantly inhibited TNF-α and CD16/32 levels in OGD-induced BV 2 microglia. There were significant differences in the results produced between the two doses of gastrodin (Fig. 5A,B).

Gastrodin Increased the Levels of CD206, IL-10, TGF-β1 and Decreased the Levels of TNF-α in LPS-Stimulated BV-2 Microglia

Western blotting analysis showed that, compared with LPS alone group, the levels of CD206, interleukin 10 (IL-10), and TGF-β1 were significantly upregulated after gastrodin treatment. At the same time, gastrodin treatment decreased the levels of TNF-α in LPS-stimulated BV-2 microglia (Fig. 5C,D). In addition, the ELISA analysis showed that the levels of TNF-α was significantly increased in LPS-stimulated BV-2 microglia, and gastrodin treatment decreased the levels of TNF-α (Supplementary Fig. 3B).

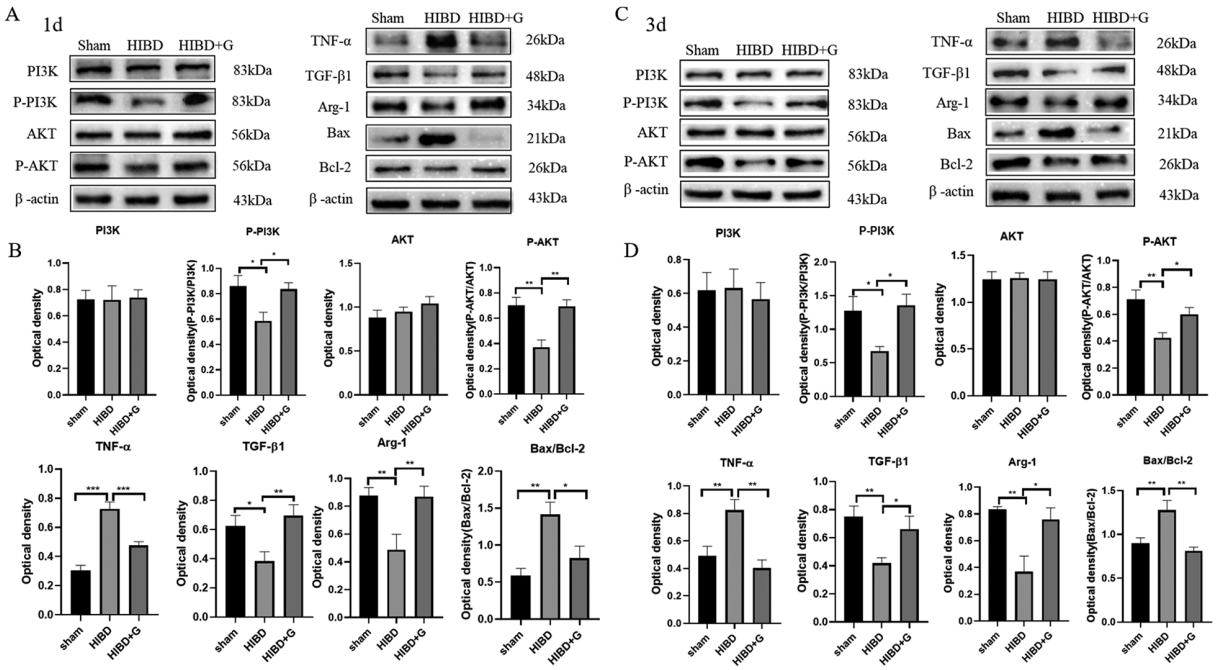

Fig. 2 Gastrodin activated the PI3K/AKT pathway, promoted the expression of M2-type microglia cell markers TGF-β1 and Arg-1, and inhibited pro-inflammatory factors TNF-α and Bax/Bcl-2. **(A, C)** Compared with those in the sham group, the levels of PPI3K, P-AKT, TGF-β1, and Arg-1 in corpus callosum microglia of HIBD mice decreased, while their levels in the Gastrodin group (HIBD + G) increased. By contrast, the levels of pro-inflammatory factors TNF-α and Bax/Bcl-2 increased significantly in the HIBD group, while Gastrodin significantly reduced their levels. **(B, D)** The protein contents in the brain tissue of mice. *P < 0.05; **P < 0.01; **P < 0.001. The values represent the mean ± SEM in triplicate

Gastrodin Promoted the PI3K/AKT Signaling Pathway in Microglia of HIBD Mice

To investigate the effect of gastrodin on the PI3K/AKT signaling pathway, western blotting was used to detect the levels of PI3K, P-PI3K, AKT, and P-AKT in corpus callosum of 1d and 3d HIBD mice treated with gastrodin. After HIBD, the levels of PI3K/AKT signaling pathway members (P-PI3K and P-AKT) in activated microglia were significantly decreased. After gastrodin treatment, the levels of P-PI3K and PAKT increased significantly. There were no significant differences in the levels of total PI3K and AKT after treatment of HIBD mice with gastrodin. The changes in the abundance of the above members of PI3K/AKT signaling pathway were similar in HIBD mice sacrificed at 1d and 3d after injury (Fig. 2).

Gastrodin Increased PI3K/AKT Signaling Pathway Protei Nlevels in OGD-Induced & LPS-Stimulated BV-2 Microglia

Western blotting analysis showed that the levels of PI3K/AKT signaling pathway members (P-PI3K and P-AKT) were significantly decreased in activated microglia treated with OGD and LPS. After gastrodin treatment, the levels of P-PI3K and P-AKT increased significantly increased, while the levels of total PI3K and AKT were not significantly different (Fig. 6A–D). Immunofluorescence labeling showed similar changes in PI3K/AKT signaling pathway members in LPS-stimulated BV-2 microglia (Fig. 6E–H).

Gastrodin Reduced Apoptosis in OGD-Induced BV-2 Microglia Through the PI3K/AKT Signaling Pathway

Western blotting analysis showed that compared with the control group, the levels of Bax/Bcl-2 in the OGD group were significantly increased, and gastrodin could markedly decreased Bax/Bcl-2 levels (Fig. 5A,B). Meanwhile, compared with those in the OGD group, Bax/Bcl-2 levels were further increased after treatment with the PI3K/AKT signaling inhibitor LY294002 (OGD + LY294002), whereas Bax/Bcl-2 levels were not significantly changed in by gastrodin and LY294002 combination treatment (OGD + LY294002 + G) (Fig. 7A). This suggests that PI3K/AKT signaling plays a role in gastrodin-mediated microglia apoptosis. However, interactions involving this pathway and other pathways should also be considered in the apoptosis of activated microglia.

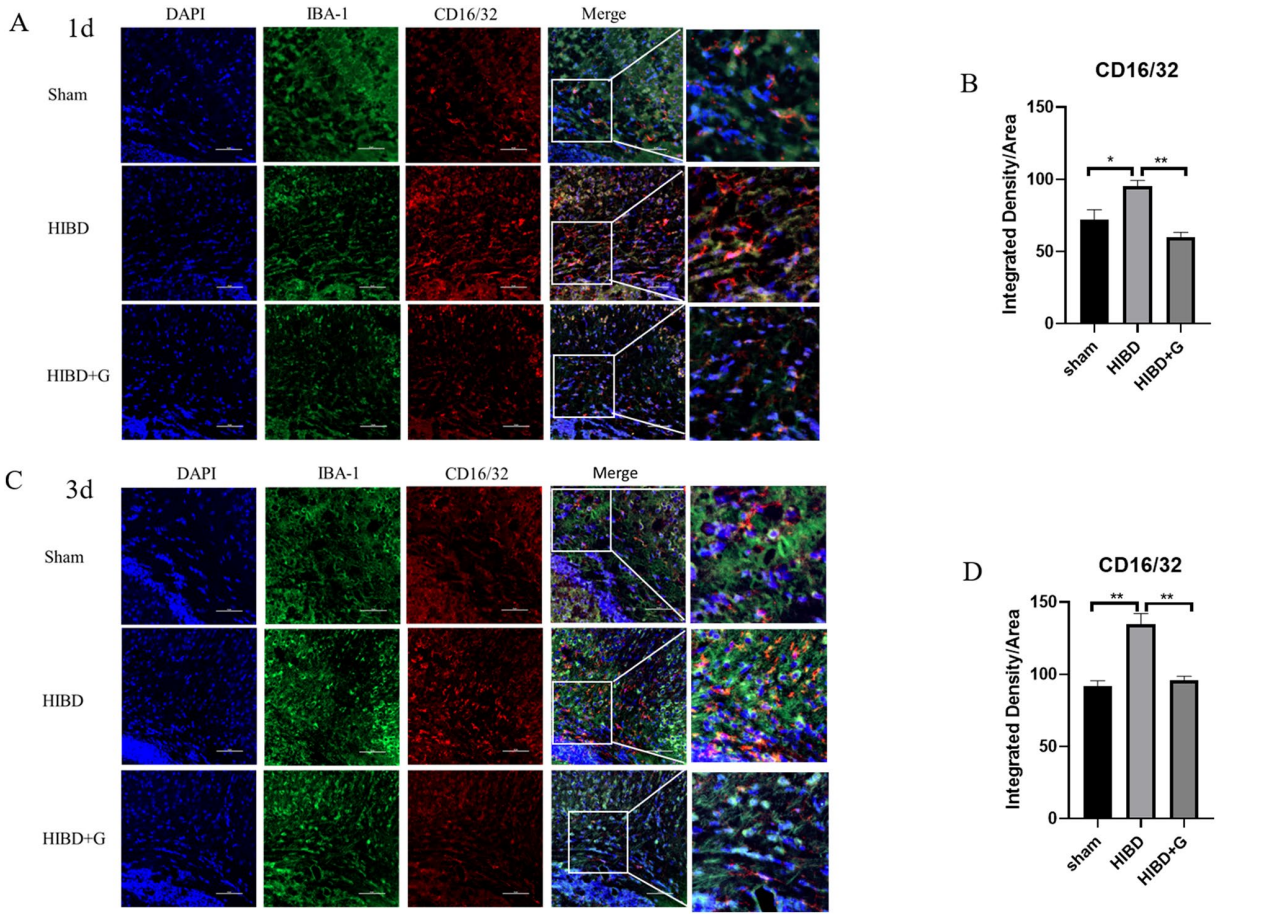

Fig. 3 Gastrodin inhibited the expression of M1-type microglia cell markers CD16/32 after HIBD. **(A, C)** Confocal microscopy image showing CD16/32 immunofluorescence (red) and its co-localization in IBA-1+ (green) microglia cells. Immunofluorescence images showing significantly increasedlevels of CD16/32 (red) in 1d and 3d IBA-1 + microglia cells (green) from HIBD mice compared with thatv in the sham group. Moreover, compared with untreated HIBD mice, the level of CD16/32 (red) in IBA-1 + microglia cells (green) of HIBD mice injected with Gastrodin at 1d and 3d was decreasedsignificantly. DAPI (blue) shows the nucleus. **(B, D)** The CD16/32 mean immunofluorescence value. *P < 0.05; **P < 0.01. Scale: 50 μm. The values represent the mean ± SEM in triplicate

Gastrodin Decreases TNF-α and Increases TGF-β1 Levels in LPS-Induced BV-2 Microglia Through the PI3K/AKT Signaling Pathway

Western blotting analysis showed that P-AKT levels in BV-2 microglia were significantly reduced after treatment with the PI3K/AKT inhibitor (LY294002) (Fig. 7B,C). Compared with those in the LPS + G group, in the L + LY294002 + G group, the levels of TNF-α in BV-2 microglia were significantly increased (Fig. 7C) and TGF-β1 levels were markedly decreased (Fig. 7D). Interestingly, compared with that in the L + LY294002 group, the combination of LY294002 and gastrodin did not exert an opposite effect on the levels of TNF-α and TGF-β1 (Fig. 7C,D). This implied that the inhibitory effect of gastrodin on inflammation acts via the PI3K/AKT pathways in LPSactivated BV-2 microglia.

The PI3K/AKT Signaling Pathway Regulates Sirt3 Expression and Gastrodin Suppressed TNF-α and Enhanced TGF-β1 in LPS-Induced BV-2 Microglia Through the PI3K/AKT-Sirt3 Pathway

After intervention using different sequences and concentrations of *Sirt3*-siRNA in BV-2 microglia, the Sirt3 mRNA and protein expression levels were significantly decreased (Fig. 8A,B). Compared with that in the LPS group, the level of Sirt3 was obviously decreased after LY294002 treatment (Fig. 7D), whereas *Akt* mRNA expression and PAKT protein levels were not significantly changed by treatment with *Sirt3*-siRNA (Fig. 8C-F). This suggested that the PI3K/AKT signaling pathway regulates Sirt3 expression in LPS-activated BV-2 microglia. In addition, western blotting analysis showed that the TNF-α protein levels were remarkably increased and TGF-β1 levels were decreased after gastrodin treatment (siRNA-1 + LPS + G) compared with those in the

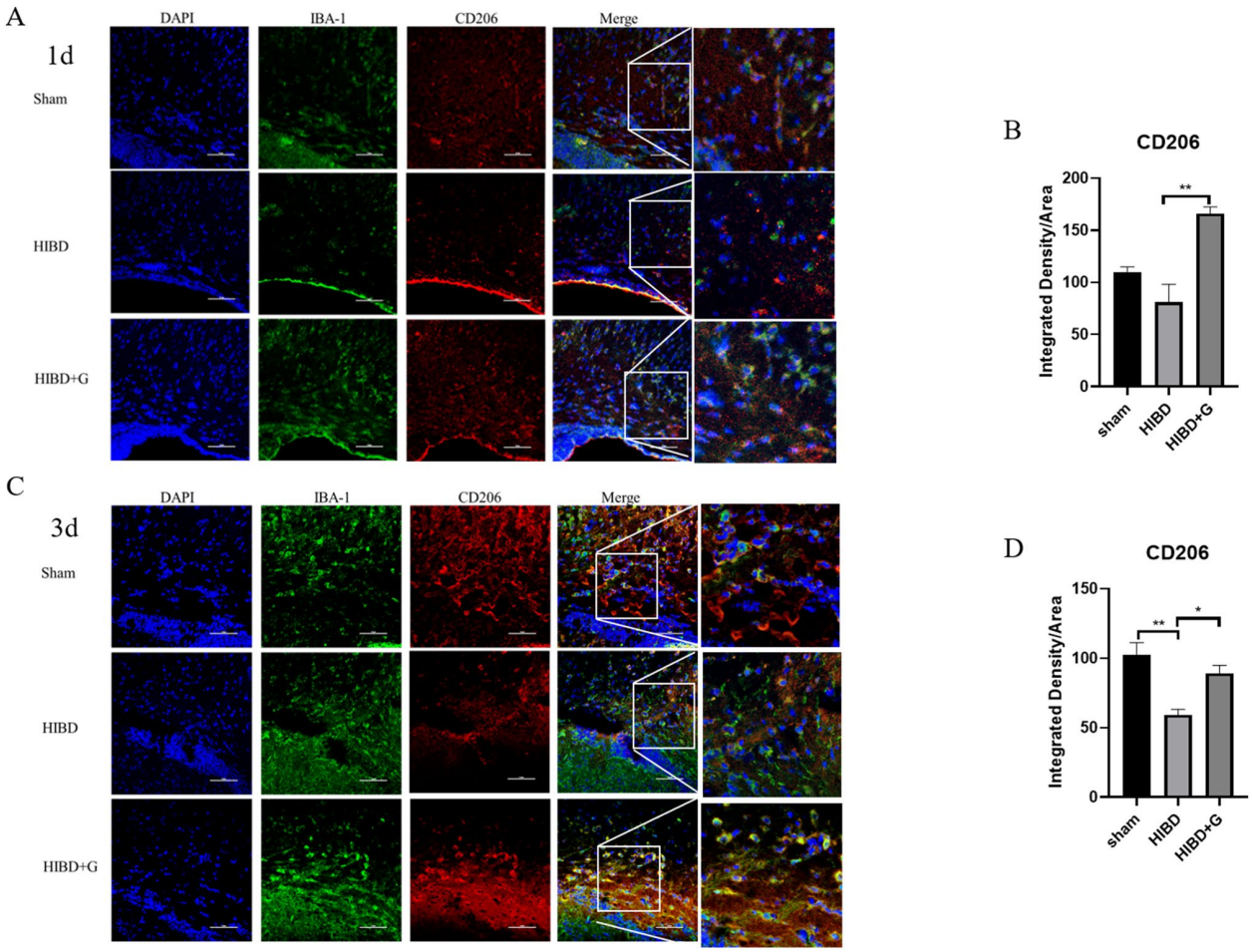

Fig. 4 Gastrodin promoted the expression of M2-type microglia cell markers CD206 after HIBD. **(A, C)** Confocal microscopy image showing CD206 immunofluorescence (red) and its co-localization in IBA-1+ (green) microglia cells. Immunofluorescence images showed significantly decreased levels of CD206(red) in 1d and 3d IBA-1 + microglia cells (green) from HIBD mice compared with that in the sham group. Moreover, compared with that in the untreated HIBD mice, the level of CD206 (red) in IBA-1 + microglia cells (green) of HIBD mice injected with Gastrodin at 1d and 3d increased significantly. DAPI (blue) shows the nucleus. **(B, D)** CD206 mean immunofluorescence value. *$P < 0.05$; **$P < 0.01$. Scale: 50 μm. The values represent the mean ± SEM in triplicate

siRNA-1 + LPS group (Fig. 8C,D). The above changes in protein levels were similar when the BV-2 microglia were treated with siRNA-2 (Fig. 8E, F). The results indicated that gastrodin could exert its anti-inflammatory role through the PI3K/AKT-Sirt3 signaling pathway.

Gastrodin Inhibits ROS Production in LPS-Activated BV-2 Microglia by Regulating P-FOXO3a via Sirt3

P-FOXO3a protein levels were decreased in LPS-activated BV-2 microglia, but were significantly increased by gastrodin pretreatment (Fig. 5C,D). Flow cytometry showed that ROS levels in BV-2 microglia increased significantly after LPS stimulation, but were decreased after gastrodin treatment. There results were significantly different between the two doses of gastrodin (Fig. 8G). In addition, compared with that in the control group, the level of P-FOXO3a in BV-2 microglia decreased significantly after *Sirt3*-siRNA intervention. However, compared with that in the *Sirt3*siRNA group, there was no significant change in the level of P-FOXO3a after gastrodin treatment (siRNA + G) and LPS stimulation (siRNA + LPS) (Fig. 8C–F). Taken together, these results suggested that Sirt3 regulates the level of P-FOXO3a; meanwhile, gastrodin might play an antioxidant role by inhibiting ROS production via Sirt3PFOXO3a signaling.

Discussion

Acute perinatal asphyxia leads to HIBD, which is an important cause of neonatal death and long-term neurodevelopmental defects [53]. Studies have shown that "selective neuronal necrosis" is the most common nerve cell damage

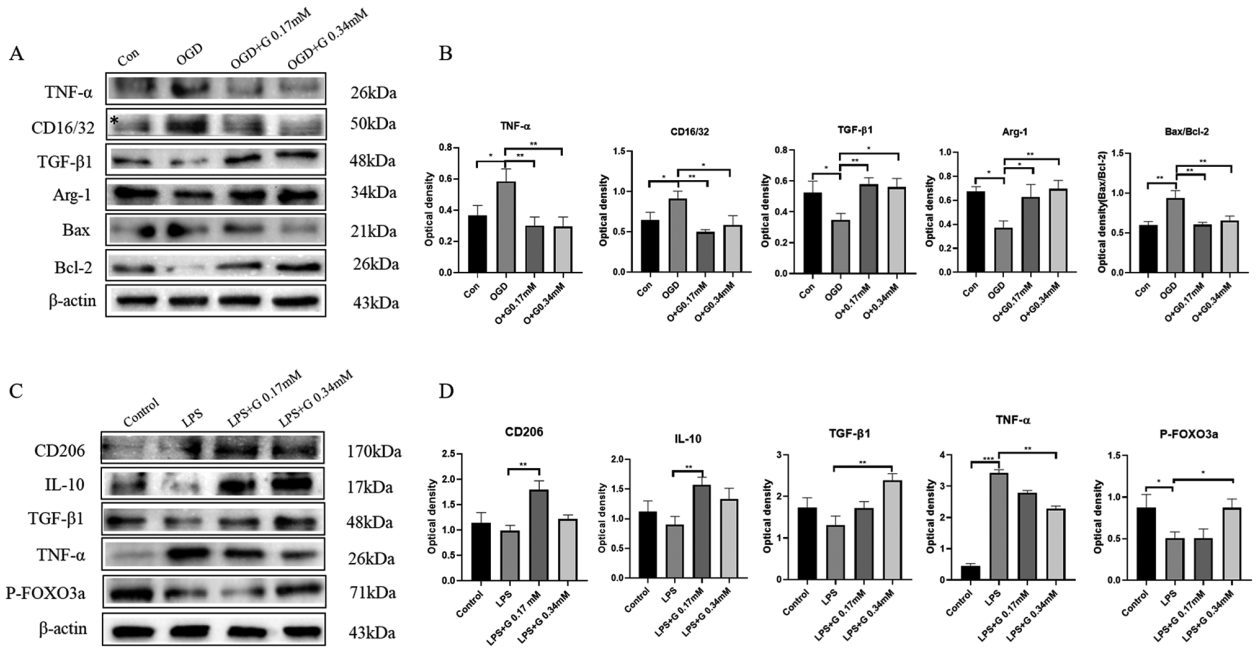

Fig. 5 Gastrodin enhanced the levels of M2-type microglia markers and P-FOXO3a in OGD and LPS-induced BV-2 microglia. Gastrodin inhibited the expression of proinflammatory cytokines TNF-α, M1-type microglia markers CD16/32, and apoptosis-related proteins (Bax/Bcl-2). **(A, C)** Western blotting showing that compared with those in the control group, the levels of TGF-β1, Arg-1, and P-FOXO3a in the OGD and LPS groups decreased, while the levels of TNF-α, CD16/32, and Bax/Bcl-2 increased. After Gastrodin treatment, the levels of TGF-β1, Arg-1, CD206, IL-10, and P-FOXO3a increased significantlyd, while the levels of TNF-α, CD16/32, and Bax/Bcl-2 decreased. The non-specific band with a *. **(B, D)** The protein content of the cells. *P < 0.05; **P < 0.01. The values represent the mean ± SEM in triplicate

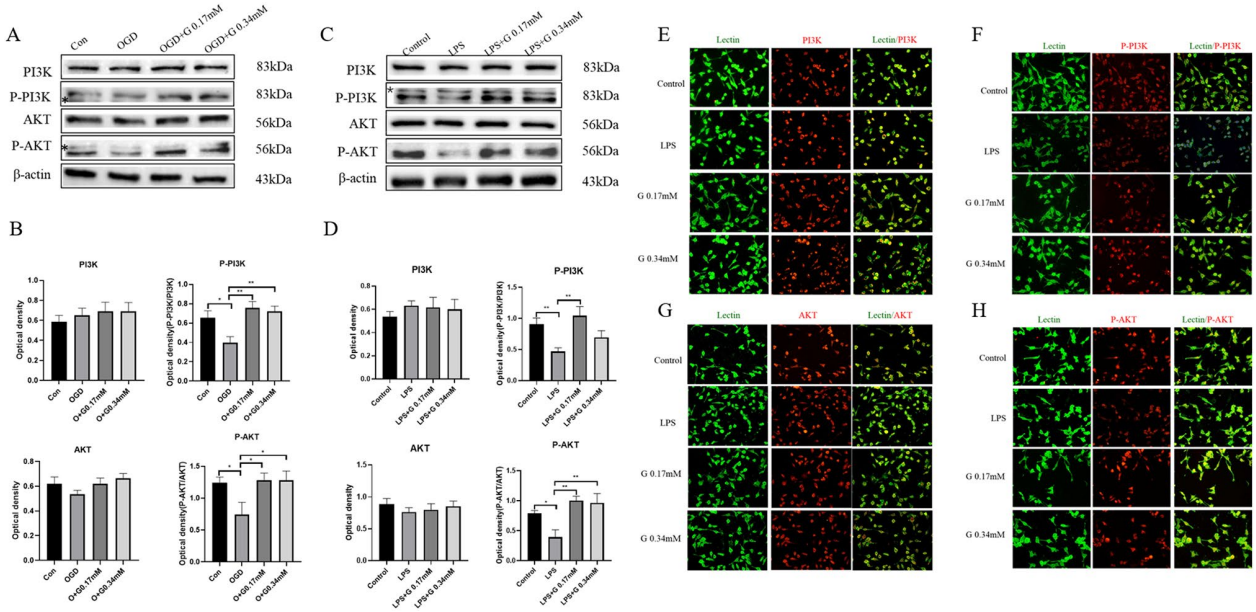

Fig. 6 Gastrodin activates the PI3K/AKT pathway in OGD-induced and LPS-activated BV-2 microglia. **(A, C)** Western blotting showing that compared with those in theOGD and LPS groups, the protein levels of P-PI3K and P-AKT in the Gastrodin group (0.17 mM and 0.34 mM) increased significantly. The non-specific band with a *. **(B, D)** The protein content in the cells. **(E-H)** Immunofluorescence images showed that the levels of P-PI3K and P-AKT (red) decreased significantly in Lectin + microglia cells (green) in the LPS group compared with that in thr control group. After Gastrodin treatment, the levels of P-PI3K and PAKT increased. Magnification: 400 ×. *P < 0.05; **P < 0.01. The values represent the mean ± SEM in triplicate

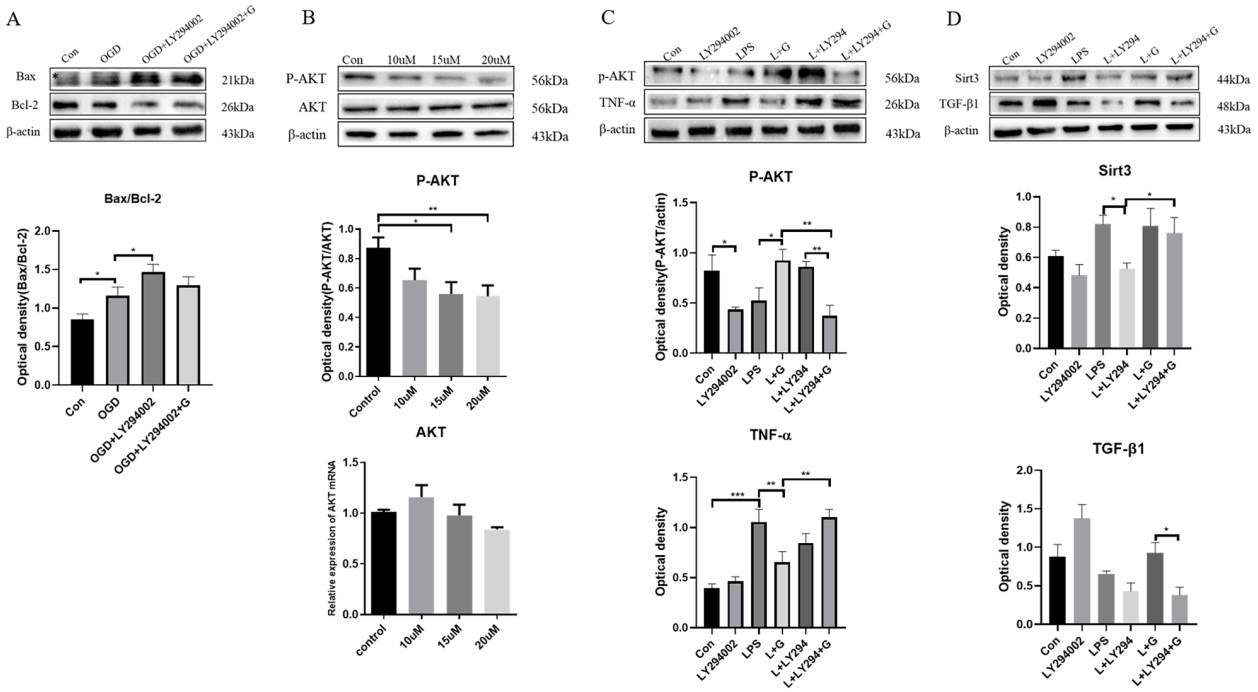

Fig. 7 Gastrodin regulated the apoptosis of activated BV-2 microglia and inhibited the expression of inflammatory mediators via the PI3K/AKT signaling pathway. **(A)** Increased levels of Bax/bcl-2 in OGD-induced BV-2 microglia compared with those in the control group. Bax/bcl-2 levels were further increased after treatment with LY294002, a PI3K/AKT pathway inhibitor, while the Bax/bcl-2 levels did not chnage significantly after treatment with the combination of Gastrodin and LY294002. The non-specific band with a *. **(B)** LY294002 (15 μM, 20 μM) inhibited P-AKT protein expression compared with that in the control. **(C)** The level of P-AKT increased in Gastrodin group compared with that in the LPS group; compared with that in the LPS + LY294002 group, the level of PAKT was decreased in the Gastrodin + LY294002 combined treatment group; compared with that in LPS + G group, the P-AKT level was decreased in the gastrodin + LY294002 combined treatment group. Compared with that in the control, the level of proinflammatory factor TNF-α was significantly increased in the LPS group and decreased in the Gastrodin group. Compared with the that in the Gastrodin treatment group, the level of TNF-α increased in the Gastrodin + LY294002 treatment group. **(D)** Compared with that in the LPS group, Sirt3 levels in LPS + LY294002 group decreased significantly, while Sirt3 levels in the Gastrodin + LY294002 combined treatment group increased significantly. At the same time, compared with that in the Gastrodin treatment group, the level of TGF-β1 in the Gastrodin + LY294002 combined treatment group decreased significantly. *P < 0.05; **P < 0.01; *** < 0.001. The values represent the mean ± SEM in triplicate

caused by hypoxic-ischemic injury in term infants [54]. Delayed neuronal death is related to excitotoxicity [55, 56], apoptosis [57, 58], and the cytotoxic effects of activated microglia [54]. Microglia are resident immune cells in the brain, and their activation is the initial link in the central nervous system (CNS) response to inflammation caused by various stimuli, including infiltration of monocytes, neutrophils, and T cells [10, 13]. Resting microglia in the healthy brain are highly active in monitoring the microenvironment of the CNS [59]. When an ischemic event occurs, microglia promote post-ischemic inflammation by producing TNF, IL-1β, ROS, and other pro-inflammatory mediators. However, they also promote the resolution of inflammation and tissue repair by producing IL-10, TGFβ, and several growth factors, including insulin like growth factor 1 (IGF-1) [60]. Studies have suggested that the activated phenotype of microglia is similar to that of macrophages, and their activation plays an important role in regulating neurogenesis in the brain [61]. Notably, microglia activation phenotypes could change from M1 (proinflammatory) to M2 (anti-inflammatory) in response to inflammatory signals during the progression of certain diseases [8, 61]. The M1 subtype is generally considered to represent effector cells that produce large amounts of pro-inflammatory cytokines. The M2 subtype is a clearly beneficial activated state associated with promoting angiogenesis, tissue remodeling, and repair [62, 63]. Therefore, inhibition of the overactivation of M1 phenotype microglia is considered an attractive therapeutic strategy to treat various neurodegenerative diseases, including neonatal HIBD, as reported in this study.

In recent years, extensive research has been conducted on the pharmacological properties of gastrodin, a natural herbal extract, which has been found to have a wide range of beneficial effects on central nervous system diseases, such as Alzheimer's disease, Parkinson's disease, and cerebral ischemia. Its mechanism of action includes antioxidant and anti-inflammatory properties, inhibition of microglia activation, and upregulation of neurotrophic factors [64]. Notably,

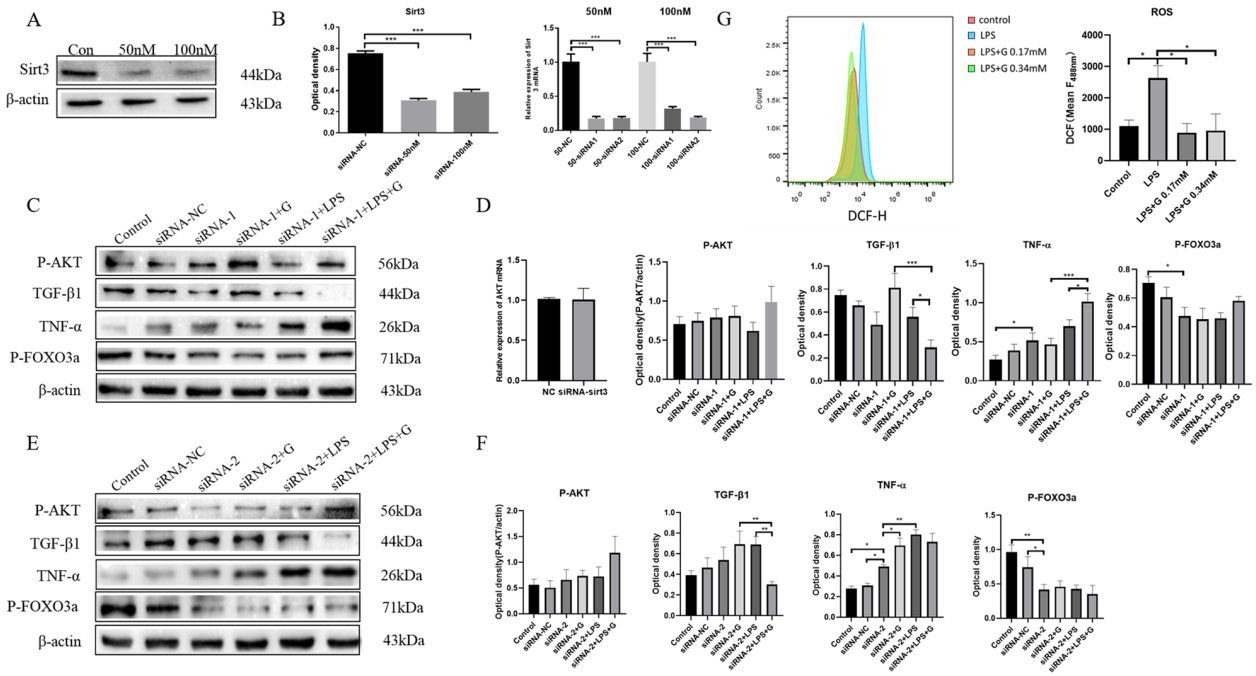

Fig. 8 Gastrodin inhibited the expression of inflammatory mediators via the PI3K/AKTSirt3 signaling pathway, might regulate the level of P-FOXO3a through Sirt3, and inhibited ROS secereton by LPS-activated BV-2 microglia. **(A)** Western blotting showing that Sirt3-siRNA (siRNA-1, siRNA-2; *Sirt3* expression was significantly decreased in the siRNA-50 nM and siRNA-100 nM groups. **(B)** Protein and mRNA levels in cells. **(C, E)** Western blotting showing no changes in P-AKT protein levels and *Akt* mRNA levels compared with the siRNA-NC group. Compared with the control group, the level of P-FOXO3a in the siRNA-1 group was significantly decreased, while the protein level of TNF-α was increased. Meanwhile, compared with that in the siRNA-1 + G group and siRNA-1 + LPS group, the protein level of TGF-β1 was significantly decreased, while the protein kevel of TNF-α was increased in siRNA-1 + LPS + G group. Compared with that in the siRNA-2 group, the above changes in protein levels were similar in the BV-2 microglia. **(D, F)** Protein and mRNA levels in cells. **(G)** Flow cytometry showing that ROS levels were significantly increased in LPSactivated BV-2 microglia compared with those in the control group, while ROS levels decreased significantly in the Gastrodin group (0.17 mM, 0.34 mM). *P < 0.05; **P < 0.01; ***P < 0.001. The values represent the mean ± SEM in triplicate

in this study, gastrodin reduced the cerebral infarct size and neuronal apoptosis, relieved neurological dysfunction after HIBD injury. Our findings also demonstrated that gastrodin increased the expressions of CD206 and Arg-1(M2 phenotype)in the corpus callosum of 1d and 3d HIBD mice and in OGD & LPS-induced microglia. In addition to its inhibitory effect on the expression of proinflammatory M1 mediators, such as TNF-α and CD16/32, gastrodin dramatically upregulated the levels of the anti-inflammatory cytokines TGF-β1 and IL-10. Thus, we concluded that gastrodin exerts its neuroprotective effect by inhibiting M1 phenotype microglia-mediated inflammatory responses.

Additionally, studies have shown that gastrodin exerts its neuroprotective effects through various signaling pathways, including the renin-angiotensin system and the Notch pathway [1] [46]. The PI3K/AKT pathway is another key pathway known to play a role in neuroprotection by decreasing the expression of proinflammatory cytokines and increasing the expression of anti-apoptotic factors [29]. In vitro studies on H9C2 cardiomyocytes demonstrated that gastrodin could inhibit the production of proinflammatory proteases and pro-inflammatory factors by activating the PI3K/AKT pathway and inhibiting mitogen activated protein kinase (MAPK) family phosphorylation. This suggested that the activation of the PI3K/AKT signaling pathway is a crucial for gastrodin to exert its anti-inflammatory effects [65]. In this study, western blotting and immunofluorescence showed that gastrodin significantly increased P-PI3K and P-AKT levels compared with those in the OGD and LPS groups. The changes in the levels of PI3K/AKT signaling pathway members in the HIBD 1d and 3d groups were consistent with the in vitro results. These findings further suggest that gastrodin attenuates microglia activation by regulating PI3K/AKT signaling. The apoptosis of microglia also plays an important role in the pathological changes of ischemic brain injury. Cerebral ischemia-reperfusion injury in mice can be alleviated by inhibiting the apoptosis of microglia [66]. In this study, apoptosis of microglia was also remarkably enhanced except the inflammatory response. Herein, the Bax/Bcl-2 ratio increased significantly in HIBD 1d and 3d mice. Similarly, in OGD-induced BV-2 microglia, Bax/Bcl-expression was also significantly increased.

However, the apoptosis of microglia activated by HIBD or OGD was alleviated after the gastrodin treatment. Interestingly, increased PI3K/AKT production and activation were observed to reduce OGD-induced cell death [67]. Therefore, we wondered whether the anti-apoptotic effect of gastrodin on microglia during HIBD was related to PI3K/AKT signaling. Western blot analysis showed that the Bax/Bcl-2 ratio increased significantly increased after pretreatment with LY294002 (a specific inhibitor of PI3K) compared with that in the OGD group. However, the Bax/Bcl-2 ratio did not change significantly in cells treated with a combination of gastrodin and LY294002 (OGD + LY294002 + G) compared with that in the OGD + LY294002 group. This further indicated that gastrodin may regulate the apoptosis of activated BV-2 microglia through the PI3K/AKT signaling pathway.

Sirtuin3 (Sirt3) is a niacinamide adenine dinucleotide (NAD)-dependent deacetylase that reduces ROS levels in primary cultures of cardiomyocytes by activating FOXO3a-dependent antioxidant genes, including those encoding manganese superoxide dismutase (MnSOD) and catalase [68]. Mitochondria are the main source of ROS and are particularly vulnerable to hypoxia and ischemia [69]. Free radicals and other oxidants are produced during cerebral ischemia, causing oxidative damage to proteins, DNA, and lipids, thus leading to neuronal death, breakdown of the blood-brain barrier, and cerebral edema [70]. In this study, ROS production was increased in LPS-activated BV-2 microglia whereas that was reduced after gastrodin treatment. Futhermore, gastrodin increased P-FOXO3a levels, which was attenuated by LPS. Therefore, we concluded that gastrodin regulates ROS production in activated microglia in a manner related to P-FOXO3a. Moreover, we pretreated LPS-activated BV-2 microglia with *Sirt3*-siRNA, and found that the level of P-FOXO3a was unchanged compared with that of gastrodin treatment. These results indicated that gastrodin possibly regulates P-FOXO3a levels through Sirt3 to reduce the production of ROS in activated microglia, thus playing a neuroprotective role.

Interestingly, there is a regulatory relationship between Sirt3 and PI3K/AKT signaling. It has been reported that Ginsenoside Rg3 promotes mitochondrial biosynthesis by regulating AKT-mechanistic target of rapamycin (mTOR)-sirtuin signal transduction [71]. Meanwhile, melatonin alleviates sodium fluoride-induced hepatotoxicity by activating PI3K/AKT-PGC-1α-Sirt3 signaling [72]. To explore the relationship between the PI3K/AKT signaling pathway and Sirt3 in microglia activated by HIBD, as well as the effect of gastrodin, the levels of PI3K/AKT signaling pathway-related proteins, Sirt3, inflammatory factor TNF-α, and trophic factor TGF-β1, were detected after blocking PI3K using LY294002. Herein, we showed that LY294002 decreased P-AKT and Sirt3 levels in LPS-activated BV-2 microglia. Morever, there was no alteration in the levels of AKT and P-AKT after *Sitr3*-siRNA intervention by qRT-PCR and western blotting analysis. This suggested that the PI3K/AKT signaling pathway can regulate Sirt3 in LPS-activated BV-2 microglia, but Sirt3 does not act on PI3K/AKT pathway reciprocally. Along with the above, we found that Sirt3 levels further increased in cells co-treated with LY294002 and gastrodin (Fig. 7D). This implied that gastrodin regulation of Sirt3 might not act exclusively through the PI3K/AKT signaling system; therefore, other pathways [1, 46] that might be involved in this effect should be considered. Notably, the level of the inflammatory cytokines TNF-α was further increased, and that of trophic factor TGF-β1 decreased after combined treatment with LY294002 and gastrodin. This indicated that inhibition of the PI3K/AKT pathway with LY294002 reversed the anti-inflammatory effect of gastrodin. Additionally, compared with that in the siRNA + LPS group, combined treatment with gastrodin decreased the abundancce of TGF-β1 in LPS-activated microglia cells, but increased the abundance of anti-inflammatory factor TNF-α. This suggested that the anti-inflammatory effect of gastrodin was neutralized by the siRNA. Taken together, gastrodin exerts its neuroprotective effect and shifts the expression of inflammatory mediators toward resolution of inflammation by modulating microglia via the PI3K/AKT-Sirt3 signaling pathway.

It's worth noting that HIBD is increasingly recognized as a sexually dimorphic disease. Clinically, male infants are more susceptible to ischemic injury than female infants and have more long-term sequelae[73, 74]. However, the mechanisms underlying this sex difference are unclear. The inflammatory process caused by the activation of microglia is the basis of cerebral ischemia pathophysiology. It is reported that under normal circumstances, the number of microglia in the newborn brain and the expression of activation markers exhibit sexual dimorphism[74]. Additionally, inflammatory phenotypes of microglia vary with age and sex[75, 76]. Interestingly, studies have reported that there is equal primary brain damage in male and female newborns during the acute phase of HIBD, which develops into sexually dimorphic outcomes at a later point in time [74]. The research has found that there are no difference in infarct volumes, IL-1β, and TNF-α levels between males and females at 1 day of neonatal hypoxic-ischemic encephalopathy (HIE), and there are no sex differences in IL-6 levels at either 1 day or 3 days of HIE[74]. Given this, our study primarily focused on the acute phase of HIBD development (i.e., 1 day and 3 days after HIBD), without examining sex differences. Moving forward, we will also take into account the impact of gender on the subsequent course of HIBD disease.

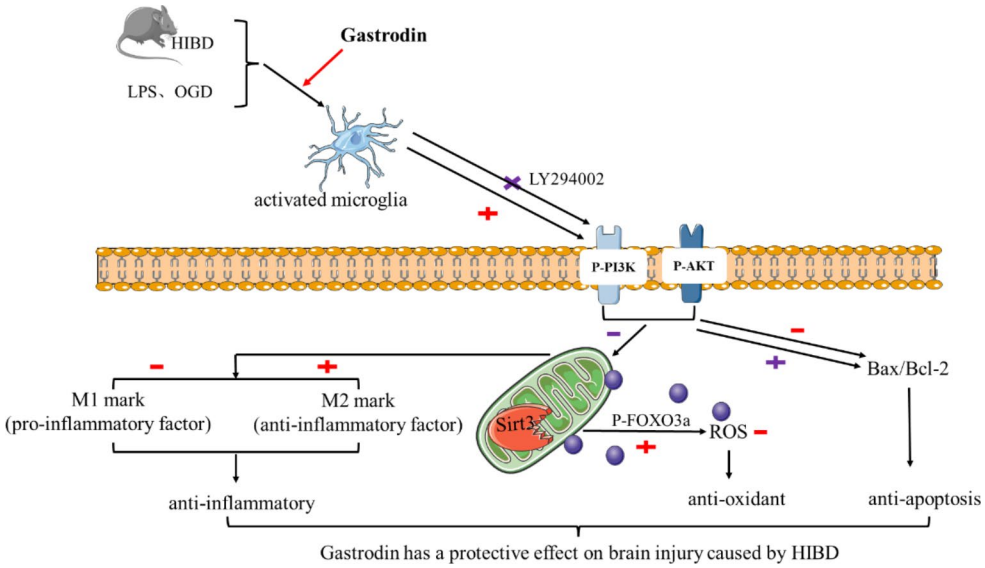

Fig. 9 Schematic diagram showing the regulatory mechanism of microglia-related factor expression changes after gastrodin pretreatment after HIBD. Gastrodin inhibits the expression of M1 microglia markers (pro-inflammatory factors) and promotes the expression of M2 microglia markers (anti-inflammatory factors) by activating PI3K/AKT-Sirt3 signaling. Gastrodin might affect the level of P-FOXO3a by activating PI3K/AKT-Sirt3 signaling, and further inhibits the release of ROS by activated microglia. Gastrodin reduces the apoptosis of microglia after HIBD by activating the PI3K/AKT pathway. In summary, gastrodin exerts neuroprotective effects via PI3K/AKT-Sirt3 signaling after HIBD.

Conclusion

Our results indicated that gastrodin has a protective effect on the brain injury caused by HIBD. Gastrodin decreased the levels of TNF-α and M1-type microglia marker CD16/32 in activated microglia cells in vitro and in vivo, while increasing the levels of trophic factor TGF-β1, anti-inflammatory factor IL-10, and the M2-type microglia markers CD206 and Arg-1. In addition, gastrodin increased the levels of PI3K/AKT signaling pathway-related proteins P-PI3K and P-AKT in activated microglia. Blocking the PI3K/AKT signaling pathway promoted the apoptosis of activated microglia cells (according to the Bax/Bcl-2 ratio) and reduced Sirt3 levels. This provides key evidence for the relationship between the PI3K/AKT signaling pathway and Sirt3 in activated microglia. The present results suggest that gastrodin can regulate the inflammatory response of microglia through the PI3K/AKT-Sirt3 pathway and reduce activated microglia apoptosis through the PI3K/AKT signaling pathway. Notably, activated microglia also showed increased ROS production, in addition to inflammation and apoptosis. We demonstrated that gastrodin could inhibit ROS production in activated microglia, the molecular mechanism of which is closely related to regulation of the Sirt3-P-FOXO3a axis (Fig. 9). However, it remains to be clarified whether gastrodin exerts antioxidant effects by directly acting on Sirt3-FOXO3a via the PI3K/AKT signaling pathway or by other means in activated microglia to regulate ROS production.

Abbreviations

HIBD	Hypoxic-ischemia brain damage
CNS	Central nervous system
Sirt3	Sirtuin3
LPS	Lipopolysaccharide
OGD	Oxygen-glucose deprivation
PI3Ks	Phosphoinositol 3 kinases
AD	Alzheimer's disease
NAD	Niacinamide adenine dinucleotide
IL-1β	Interleukin-1β
TNF-α	Tumor necrosis factor-α
iNOS	Inducible nitric oxide synthase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
BCA	Bicinchoninic acid
ROS	Reactive oxygen species
M1	Pro-inflammatory
M2	Anti-inflammatory
LY294002	PI3K/AKT pathway inhibitor
FOXO3a	Forkhead box O3
CD	Cluster of differentiation
DAPI	6-diamidino-2-phenylindole
IBA-1	Ionized calcium-binding adaptor molecule
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
TTC	2,3,5-Triphenyltetrazolium chloride staining

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Author Contributions J-JL conceptualized and designed this study. J-JL supervised the whole project. H-JZ, P-XW, X-QR, H-LS, J-SS, TG and C-W carried out the experiments. H-JZ performed acquisition and analysis of data. H-JZ and J-JL prepared the manuscript. All authors read and approved the final manuscript.

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Data Availability All data supporting this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate All animal procedures were approved and conducted in accordance with the requirements of the Ethics Committee of Kunming Medical University (kmmu20211454).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing interests The authors declare that they have no competing interests.

References

1. Guo J, Zhang X L, Bao Z R et al (2021) Gastrodin regulates the Notch Signaling Pathway and Sirt3 in activated Microglia in Cerebral hypoxic-ischemia neonatal rats and in activated BV-2 microglia [J]. Neuromolecular Med 23(3):348–362
2. Li SJ, Liu W, Wang JL et al (2014) The role of TNF-α, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy [J]. Eur Rev Med Pharmacol Sci 18(6):905–909
3. Huang J Z, Ren Y, Jiang Y et al (2018) GluR1 protects hypoxic ischemic brain damage via activating akt signaling pathway in neonatal rats [J]. Eur Rev Med Pharmacol Sci 22(24):8857–8865
4. Jellema R K, Lima Passos V, Zwanenburg A et al (2013) Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep [J]. J Neuroinflammation 10:13
5. Guo L, Wang D, BO G et al (2016) Early identification of hypoxic-ischemic encephalopathy by combination of magnetic resonance (MR) imaging and proton MR spectroscopy [J]. Experimental and Therapeutic Medicine 12(5):2835–2842
6. Yang L, Zhao H (2020) Treatment and new progress of neonatal hypoxic-ischemic brain damage [J]. Histol Histopathol 35(9):929–936
7. Du Plessis A J, Volpe JJ (2002) Perinatal brain injury in the preterm and term newborn [J]. Curr Opin Neurol 15(2):151–157
8. Liu F, Mccullough LD (2013) Inflammatory responses in hypoxic ischemic encephalopathy [J]. Acta Pharmacol Sin 34(9):1121–1130
9. Kreutzberg G W (1996) Microglia: a sensor for pathological events in the CNS [J]. Trends Neurosci 19(8):312–318
10. Yenari M A, Kauppinen T M, Swanson RA (2010) Microglial activation in Stroke: therapeutic targets [J]. Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics 7(4):378–391
11. El Khoury J, Hickman S E, Thomas C A et al (1998) Microglia, scavenger receptors, and the pathogenesis of Alzheimer's Disease [J]. Neurobiol Aging 19(1 Suppl):S81–S84
12. Thomas W E (1992) Brain macrophages: evaluation of microglia and their functions [J]. Brain Res Brain Res Rev 17(1):61–74
13. Zheng Z, Yenari MA (2004) Post-ischemic inflammation: molecular mechanisms and therapeutic implications [J]. Neurol Res 26(8):884–892
14. Davies C A, Loddick S A, Stroemer R P et al (1998) An integrated analysis of the progression of cell responses induced by permanent focal middle cerebral artery occlusion in the rat [J]. Exp Neurol 154(1):199–212
15. Schroeter M, Jander S, Witte O W et al (1994) Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion [J]. J Neuroimmunol 55(2):195–203
16. Walker D G, Lue L F (2015) Immune phenotypes of microglia in human neurodegenerative Disease: challenges to detecting microglial polarization in human brains [J]. Alzheimers Res Ther 7(1):56
17. Perego C, Fumagalli S, De Simoni M G (2011) Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice [J]. J Neuroinflammation 8:174
18. Fumagalli S, Perego C, Ortolano F et al (2013) CX3CR1 deficiency induces an early protective inflammatory environment in ischemic mice [J]. Glia 61(6):827–842
19. He Y, Gao Y, Zhang Q et al (2020) IL-4 switches Microglia/macrophage M1/M2 polarization and alleviates neurological damage by modulating the JAK1/STAT6 pathway following ICH [J]. Neuroscience 437:161–171
20. Tang Y (2016) Differential roles of M1 and M2 microglia in neurodegenerative Diseases [J]. Mol Neurobiol 53(2):1181–1194
21. Kaur C, Rathnasamy G, Ling EA (2013) Roles of activated microglia in hypoxia induced neuroinflammation in the developing brain and the retina [J]. J Neuroimmune Pharmacol 8(1):66–78
22. Del Bigio M R, Becker LE (1994) Microglial aggregation in the dentate gyrus: a marker of mild hypoxic-ischaemic brain insult in human infants [J]. Neuropathol Appl Neurobiol 20(2):144–151
23. Disdier C, Stonestreet BS (2020) Hypoxic-ischemic-related cerebrovascular changes and potential therapeutic strategies in the neonatal brain [J]. J Neurosci Res 98(7):1468–1484
24. Pappas A, Shankaran S, Mcdonald S A et al (2015) Cognitive outcomes after neonatal encephalopathy [J]. Pediatrics 135(3):e624–e634
25. Gluckman P D, Wyatt JS (2005) Selective head cooling with mild systemic Hypothermia after neonatal encephalopathy: multicentre randomised trial [J]. Lancet (London England) 365(9460):663–670
26. Kim HJ, Moon K D, Oh S Y et al (2001) Ether fraction of methanol extracts of Gastrodia elata, a traditional medicinal herb,

protects against kainic acid-induced neuronal damage in the mouse hippocampus [J]. Neuroscience letters, 314(1–2): 65 – 8
27. Dai J N, Zong Y, Zhong L M et al (2011) Gastrodin inhibits expression of inducible NO synthase, cyclooxygenase-2 and pro-inflammatory cytokines in cultured LPS-stimulated microglia via MAPK pathways [J]. PLoS ONE 6(7):e21891
28. Peng Z, Wang S, Chen G et al (2015) Gastrodin alleviates cerebral ischemic damage in mice by improving anti-oxidant and anti-inflammation activities and inhibiting apoptosis pathway [J]. Neurochem Res 40(4):661–673
29. Li X, Zhang J, Zhu X et al (2015) Progesterone reduces inflammation and apoptosis in neonatal rats with hypoxic ischemic brain damage through the PI3K/Akt pathway [J]. Int J Clin Exp Med 8(5):8197–8203
30. Narayanankutty A (2019) PI3K/ Akt/ mTOR pathway as a therapeutic target for Colorectal Cancer: a review of preclinical and clinical evidence [J]. Curr Drug Targets 20(12):1217–1226
31. Kamada H, Nito C, Endo H et al (2007) Bad as a converging signaling molecule between survival PI3-K/Akt and death JNK in neurons after transient focal cerebral ischemia in rats [J]. J Cereb Blood Flow Metab 27(3):521–533
32. Ma X H, Gao Q, Jia Z et al (2015) Neuroprotective capabilities of TSA against cerebral ischemia/reperfusion injury via PI3K/Akt signaling pathway in rats [J]. Int J Neurosci 125(2):140–146
33. Yang W, Liu Y, Xu Q Q et al (2020) Sulforaphene Ameliorates Neuroinflammation and Hyperphosphorylated Tau Protein via Regulating the PI3K/Akt/GSK-3β Pathway in Experimental Models of Alzheimer's Disease [J]. Oxid Med Cell Longev, 2020: 4754195
34. Fu X, Chen H (2020) C16 peptide and angiopoietin-1 protect against LPS-induced BV-2 microglial cell inflammation [J]. Life Sci 256:117894
35. Zhang B, Yang N, Mo ZM et al (2017) IL-17A enhances microglial response to OGD by regulating p53 and PI3K/Akt pathways with involvement of ROS/HMGB1 [J]. Front Mol Neurosci 10:271
36. North B J Verdine, Sirtuins (2004) Sir2-related NAD-dependent protein deacetylases [J]. Genome Biol 5(5):224
37. Li Y, Ma Y, Song L et al (2018) SIRT3 deficiency exacerbates p53/Parkin–mediated mitophagy inhibition and promotes mitochondrial dysfunction: implication for aged hearts [J]. Int J Mol Med 41(6):3517–3526
38. Jing E, Emanuelli B, Hirschey MD et al (2011) Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production [J]. Proc Natl Acad Sci U S A 108(35):14608–14613
39. Haigis M C, Deng C X, Finley L W et al (2012) SIRT3 is a mitochondrial Tumor suppressor: a scientific tale that connects aberrant cellular ROS, the Warburg effect, and carcinogenesis [J]. Cancer Res 72(10):2468–2472
40. Alhazzazi T Y, Kamarajan P (2011) SIRT3 and cancer: Tumor promoter or suppressor? [J]. Biochim Biophys Acta 1816(1):80–88
41. Ahn B H, Kim H S Songs et al (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis [J]. Proc Natl Acad Sci U S A 105(38):14447–14452
42. Wang X, Dai Y, Zhang X et al (2021) CXCL6 regulates cell permeability, proliferation, and apoptosis after ischemia-reperfusion injury by modulating Sirt3 expression via AKT/FOXO3a activation [J], vol 22. Cancer biology & therapy, pp 30–39. 1
43. Wang Z, Li Y, Wang Y et al (2019) Pyrroloquinoline quinine protects HK-2 cells against high glucose-induced oxidative stress and apoptosis through Sirt3 and PI3K/Akt/FoxO3a signaling pathway [J]. Biochem Biophys Res Commun 508(2):398–404
44. Semple BD, Blomgren K, Gimlin K et al (2013) Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species [J]. Prog Neurobiol 106–107:1–16
45. Min Y, Yan L, Wang Q et al (2020) Distinct residential and infiltrated macrophage populations and their phagocytic function in mild and severe neonatal hypoxic-ischemic brain damage [J]. Front Cell Neurosci 14:244
46. Liu SJ, Liu X Y, Li JH et al (2018) Gastrodin attenuates microglia activation through renin-angiotensin system and Sirtuin3 pathway [J]. Neurochem Int 120:49–63
47. Li C, Mo Z, Lei J et al (2018) Edaravone attenuates neuronal apoptosis in hypoxic-ischemic brain damage rat model via suppression of TRAIL signaling pathway [J]. Int J Biochem Cell Biol 99:169–177
48. Liu X H, Yan H, Xu M et al (2013) Hyperbaric oxygenation reduces long-term brain injury and ameliorates behavioral function by suppression of apoptosis in a rat model of neonatal hypoxia-ischemia [J]. Neurochem Int 62(7):922–930
49. Huang J, Lu W, Doycheva D M et al (2020) IRE1alpha inhibition attenuates neuronal pyroptosis via miR-125/NLRP1 pathway in a neonatal hypoxic-ischemic encephalopathy rat model [J]. J Neuroinflammation 17(1):152
50. Li JJ, Lu J, Kaur C et al (2009) Expression of angiotensin II and its receptors in the normal and hypoxic amoeboid microglial cells and murine BV-2 cells [J]. Neuroscience 158(4):1488–1499
51. Livak K J, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method [J]. Methods 25(4):402–408
52. Rangarajan P, Karthikeyan A, LU J et al (2015) Sirtuin 3 regulates Foxo3a-mediated antioxidant pathway in microglia [J]. Neuroscience 311:398–414
53. Hasegawa M, Ogihara T, Tamai H et al (2009) Hypothermic inhibition of apoptotic pathways for combined neurotoxicity of iron and ascorbic acid in differentiated PC12 cells: reduction of oxidative stress and maintenance of the glutathione redox state [J]. Brain Res 1283:1–13
54. Inder T E, Volpe JJ (2000) Mechanisms of perinatal brain injury [J]. Semin Neonatol 5(1):3–16
55. Tan W K, Williams C E, During M J et al (1996) Accumulation of cytotoxins during the development of seizures and edema after hypoxic-ischemic injury in late gestation fetal sheep [J]. Pediatr Res 39(5):791–797
56. Tan W K, Williams C E, Gunn A J et al (1992) Suppression of postischemic epileptiform activity with MK-801 improves neural outcome in fetal sheep [J]. Ann Neurol 32(5):677–682
57. Macmanus JP, Buchan A M, Hill I E et al (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain [J]. Neurosci Lett 164(1–2):89–92
58. Beilharz E J, Williams C E, Dragunow M et al (1995) Mechanisms of delayed cell death following hypoxic-ischemic injury in the immature rat: evidence for apoptosis during selective neuronal loss [J]. Brain Res Mol Brain Res 29(1):1–14
59. Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo [J]. Science 308(5726):1314–1318
60. Iadecola C, Anrather J (2011) The immunology of Stroke: from mechanisms to translation [J]. Nat Med 17(7):796–808
61. Varnum MM (2012) The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's Disease brain [J]. Arch Immunol Ther Exp (Warsz) 60(4):251–266
62. Porta C, Rimoldi M, Raes G et al (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB [J]. Proc Natl Acad Sci U S A 106(35):14978–14983
63. Mantovani A, Sozzani S, Locati M et al (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for

polarized M2 mononuclear phagocytes [J]. Trends Immunol 23(11):549–555
64. Liu Y, Gao J (2018) A review on Central Nervous System effects of Gastrodin [J]. Front Pharmacol 9:24
65. Yang P, Han Y (2013) Gastrodin attenuation of the inflammatory response in H9c2 cardiomyocytes involves inhibition of NF-κB and MAPKs activation via the phosphatidylinositol 3-kinase signaling [J]. Biochem Pharmacol 85(8):1124–1133
66. Cheng C, Chen X, Wang Y et al (2021) MSCs–derived exosomes attenuate ischemia-reperfusion brain injury and inhibit microglia apoptosis might via exosomal miR-26a-5p mediated suppression of CDK6 [J]. 27(1):67 Molecular medicine (Cambridge, Mass)
67. Huang Y, Yu J, Wan F et al (2014) Panaxatriol saponins attenuated oxygen-glucose deprivation injury in PC12 cells via activation of PI3K/Akt and Nrf2 signaling pathway [J]. Oxid Med Cell Longev, 2014: 978034
68. Sundaresan N R, Gupta M, Kim G et al (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice [J]. J Clin Invest 119(9):2758–2771
69. Wang B, Guo H, Li X et al (2018) Adiponectin attenuates oxygen-glucose Deprivation-Induced mitochondrial oxidative Injury and apoptosis in hippocampal HT22 cells via the JAK2/STAT3 pathway [J]. Cell Transpl 27(12):1731–1743
70. Moro MA, Almeida A, Bolanos J P et al (2005) Mitochondrial respiratory chain and free radical generation in Stroke [J]. Free Radic Biol Med 39(10):1291–1304
71. Yang K E, Jang H J, Hwang I H et al (2020) Stereoisomer-specific ginsenoside 20(S)-Rg3 reverses replicative senescence of human diploid fibroblasts via Akt-mTOR-Sirtuin signaling [J]. J Ginseng Res 44(2):341–349
72. Song C, Zhao J, Fu B et al (2017) Melatonin-mediated upregulation of Sirt3 attenuates sodium fluoride-induced hepatotoxicity by activating the MT1-PI3K/AKT-PGC-1α signaling pathway [J]. Free Radic Biol Med 112:616–630
73. Ai Mamun A, Yu H (2018) Inflammatory responses are sex specific in chronic hypoxic-ischemic encephalopathy [J]. Cell Transpl 27(9):1328–1339
74. Mirza MA, Ritzel R, Xu Y et al (2015) Sexually dimorphic outcomes and inflammatory responses in hypoxic-ischemic encephalopathy [J]. J Neuroinflammation 12:32
75. Ngwa C, Qi S, Mamun A A et al (2021) Age and sex differences in primary microglia culture: a comparative study [J]. J Neurosci Methods 364:109359
76. Qi S, Al Mamun A, Ngwa C et al (2021) X chromosome escapee genes are involved in ischemic sexual dimorphism through epigenetic modification of inflammatory signals [J]. J Neuroinflammation 18(1):70