Original Article



# Gastrodin exerts protective effects in reactive TNC1 astrocytes via regulation of the Notch signaling pathway

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**Background:** Gastrodin (GAS) has been proven to play a therapeutic role in a variety of neurological diseases by affecting activated astrocytes, however, the underlying mechanisms have not been fully illustrated. This study aimed to investigate if GAS exerts the neuroprotective effect through regulating the Notch signaling pathway involved in reactive astrocytes.

**Methods:** Astrocyte cell lines (TNC1 cells) were cultured *in vitro*. The hypoxic-ischemic cell model was prepared using the oxygen-glucose deprivation (OGD) method, GAS's pretreatment concentration was 0.34 mM, intervention for 1 hour. Cell counting kit-8 (CCK-8) assay, Transwell migration assay, immunofluorescent staining (double staining), and Western blotting were used to observe the effects of OGD or GAS interference on the function of astrocytes, and the changes of key protein expressions in the Notch signaling pathway were analyzed.

**Results:** GAS had no obvious toxic effect on TNC1 astrocytes under physiological conditions. Following OGD, GAS can not only improve cell viability and migration, but also regulate the production of inflammatory mediators. We also found that OGD significantly increased the expression of key proteins related to the Notch signaling pathway, Notch-1, intracellular Notch receptor domain (NICD), recombining binding protein suppressor of hairless (RBP-JK), transcription factor hairy and enhancer of split-1 (Hes-1) in TNC1 astrocytes, which was significantly inhibited by GAS. In addition, GAS inhibited the OGD-induced expression of TNC1 astrocyte tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), and enhanced the expression of nutrient factors, including brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1). The Notch signaling pathway specific inhibitor, N-[N-(3,5-Difluorophenacetyl)-1-alany1]-S-phenyglycine t-butylester (DAPT), could significantly enhance the effect of GAS on TNC1 astrocytes after OGD, such as the inhibition of inflammatory factors and the up-regulation of neurotrophic factors.

**Conclusions:** GAS exerts dual effects on astrocytes via regulation of the Notch signaling pathway. We found that it could inhibit the pro-inflammatory factors mediated by astrocytes, and also promote the secretion of neurotrophic factors by astrocytes. These results provide a new biological mechanism for the treatment of neuroinflammatory diseases by GAS.

**Keywords:** Gastrodin (GAS); astrocytes; Notch signaling pathway; biological mechanism

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## Introduction

Perinatal asphyxia often results in hypoxic ischemic brain damage (HIBD), which is characterized by cerebral hypoxia and reduced or suspended cerebral blood flow (1). HIBD is strongly associated with acute neonatal death and subsequent lifelong neurological deficits, accounting for approximately 23% of neonatal deaths each year (2). Considering the major advances in modern technology, prenatal and neonatal care have improved over the past few decades, which has reduced the risk of death, and neurodevelopmental disorders such as cerebral palsy, hearing loss, and other neuromotor disorders have also declined significantly (3). However, approximately 50% of newborn are still at risk of neurological sequelae and even death (4). Therefore, the development of safer and more effective therapies for the treatment of neonatal HIBD is urgently needed.

Previous studies have shown that the pathological mechanisms of neonatal HIBD are relatively complex, involving in mitochondrial damage, inflammation, oxidative stress and progressive neuronal cell death (5). With the progression of the disease, neuroinflammation, loss of mitochondrial permeability, and dysfunction of brain automatic regulation will occur, as well as other brain injury manifestations (6). Some scholars have confirmed that continuous inflammation may aggravate brain injury (7). Therefore, reducing cellular oxidative stress injury and the inflammatory response, and improving the neuronal survival rate may be the most effective treatment strategies for neonatal HIBD.

Neurons are easily damaged by ischemia and hypoxia, while damage to supporting glial cells (such as astrocytes) may lead to secondary neuronal damage (8). Astrocytes, as the essential players in the central nervous system (CNS), have many vital functions for CNS development and homeostasis, including modulation of neuronal communication, as well as participation in CNS repair and damage in the context of disease and injury (9,10). Under conditions of ischemia and hypoxia insult, reactive astrocytes secrete reactive oxygen species, pro-inflammatory cytokines, interleukins (ILs), and matrix metalloproteinases, thereby mediating the inflammatory response process to exhibit adverse effects (11-13). On the other hand, in brain injuries, reactive astrocytes can clear cellular debris, induce glial scar formation and release of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) for neuronal survival or the maintenance of cellular microenvironmental homeostasis (14). Hence, loss-of-function astrocytes represent an important adverse factor for recovery and repair of the brain after neonatal hypoxic-ischemic injury (15). A new perspective proposes that reactive astrocytes may play a conjoining regulatory role as a key point or hub of neuroinflammation in CNS disease (16,17).

Gastrodin (GAS) with a molecular formula of $C_{13}H_{18}O_7$ and a molecular weight of 286 Da, as the second compound identified from Tian Ma, which was isolated in 1958 (18), is a biologically active component and widely used in Chinese medicine (19). At present, GAS is widely used in the clinical treatment of neurological diseases such as neurasthenia, dizziness, epilepsy, and vertebrobasilar artery insufficiency (20) due to its anti-inflammatory effect. A recent report confirmed GAS exerts anti-inflammatory effects via signal transducers and activators of transcription-3 (STAT3) and nuclear factor κB (NF-κB) signaling pathways in reactive astrocytes in middle cerebral artery occlusion (MCAO) rat (21). However, whether GAS has protective effect in HIBD and the underlying mechanism have not yet been elucidated. Our previous studies have confirmed that ischemia and hypoxia after HIBD induce the activation of the Notch signaling pathway, and inhibition of the Notch signaling pathway can improve neurological deficits after HIBD (22). The Notch pathway , as an evolutionarily conserved signaling pathway, has a prominent role in regulating the proliferation, differentiation and apoptosis of neural progenitor cells (23). Notch-1 has been reported as a key factor required for reactive astrocyte activation in the infarct region as well as worsening brain damage and functional outcome after stroke (24). Study was found that irisin could alleviate post-ischemic inflammation, neuronal apoptosis and improve neurological dysfunction through regulating the Notch signaling pathway (25), suggesting that Notch signaling pathway may be a potential therapeutic target for HIBD. Therefore, this study aimed to observe the effect of GAS intervention on astrocytes, and investigate whether GAS exerts its protective effects through the Notch signaling pathway in reactive astrocyte inducing by oxygen-glucose deprivation (OGD) method, which is commonly used to mimic cerebral ischemia *in vitro* research (26). We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5787).

## Methods

### *Cell culture, main reagents, and instruments*

Astrocyte cell lines (TNC1 cells) was donated by the Department of Human Anatomy, Histology and Embryology of the National University of Singapore

 

(ATCC, USA, CRL-2005$^{TM}$), and was cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium that containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin double antibody. The high-glucose DMEM medium, sugar-free medium DMEM, penicillin/streptomycin double antibody, and FBS were purchased from BI Company (Israel). GAS (purity 99%) was purchased from Sigma (SMB00313, USA). The bicinchoninic acid (BCA) kit was purchased from Meilun Biotech (China). The polyvinylidene fluoride (PVDF) membrane and chemiluminescence reagent were purchased from Millipore (USA). The anoxic chamber cell incubator was purchased from Billups-Rothenberg (USA).

### *OGD model establishment*

Cell culture was cultured to the third generation. TNC1 cells were inoculated at a density of $3\times10^6$/mL and randomly divided into three groups: a normal control group (CON group), an oxygen glucose deprivation model group (OGD group), and a GAS pre-treated group (GAS + O group). The GAS + O group was pretreated with 0.34 mM GAS for 1 h prior to OGD. Before hypoxia, the basic medium of the OGD group and the GAS + O group was replaced with serum-free and sugar-free DMEM medium and placed in the hypoxia chamber. The mixture [carbon dioxide ($CO_2$) 5%, nitrogen ($N_2$) 95%] was filled at 37 ℃ for hypoxia treatment. Four hours later, the cells were taken out for further experimentation.

### *GAS concentration determination*

TNC1 cells were co-cultured with the gradient concentrations (0.17, 0.34, 0.51, 0.68, 0.85, 1.02 mM) of GAS for 1 h, and then the cell viability was detected to observe whether GAS with different concentration had toxic effect on TNC1 cells. This test was repeated three times, and the average value was taken for statistical analysis.

### *TNC1 cell viability detection*

TNC1 cells were divided into control, OGD, and GAS + O groups in a 96-well plate. The treatment method was performed as previously mentioned. After hypoxia treatment for 4 h, the cells were taken out and incubated in cell counting kit-8 (CCK-8) solution under dark conditions (CCK-8 solution: medium =1:100), and then incubated in an incubator at 37 ℃ for 2 h. Finally, the corresponding absorbance [optical density (OD)] value of the cells was read at a wavelength of 450 nm using the microplate analyzer. This test was repeated three times, and the average value was taken for statistical analysis.

### *Western blotting test*

After hypoxia, TNC1 astrocytes in the different groups (CON, OGD, GAS + O) were washed twice with phosphate buffered saline (PBS) and then treated with lysate buffer. The cell lysates were extracted and centrifuged at 14,000 rpm/min for 15 min to collect the supernatant. Protein concentration was tested by BCA protein assay (Melon, China). After sample loading, electrophoresis and transfer, 5% skim milk was used for sealing, then the bands were immersed in the primary antibody and placed in a 4 ℃ shaker overnight. The primary antibodies were as follows: glial fibrillary acidic protein (GFAP) (monoclonal, 1:1,000, MAB360, Millipore, USA), Notch-1 (rabbit polyclonal 1:1,000, ab52627, Abcam, UK), intracellular Notch receptor domain (NICD, rabbit polyclonal 1:1,000, ab8925, Abcam, UK), recombining binding protein suppressor of hairless (RBP-JK) (rabbit polyclonal 1:100, ab180588, Abcam, UK), transcription factor hairy and enhancer of split-1 (Hes-1) (mouse polyclone 1:500, SC-166410, Santa Cruz, UK); BDNF (rabbit monoclonal 1:1,000, ab108139, Abcam, UK), insulin-like growth factor-1 (IGF-1) (rabbit polyclonal 1:1,000, orb10886, Biorbyt, UK); Sirtuin 3 (Sirt3, mouse polyclone 1:500, SC-365175, Santa Cruz, USA); tumor necrosis factor-α (TNF-α) (rabbit polyclonal antibody, 1:1,000, AB1837P, Millipore, USA); and mouse anti-β-actin monoclonal antibody (1:5,000, Proteintech, China). The next day, after the secondary antibody was incubated and washed, ultra-sensitive efficient chemiluminescence kit (ECL) luminescent reagent was used to expose, develop, and store the strips in a dark room at room temperature. The strips were analyzed with Image J software (version 1.8.0, National Institute of Mental Health, USA), repeated three times, and the average value was taken for statistical analysis.

### *Immunofluorescence staining*

Immunofluorescence staining was used to detect the expression of TNF-α, BDNF, IGF-1, Sirt3, and Notch signaling proteins in TNC1 astrocytes after OGD. TNC1 cells were inoculated on the cell slides at a density of $5\times10^4$/mL and divided into three groups: CON, OGD, and GAS + O. The treated method was performed as described

 

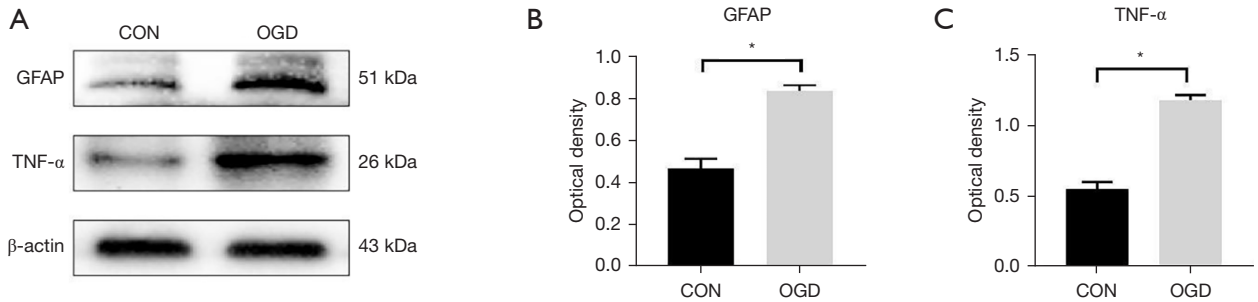


**Figure 1** The protein expressions of GFAP and TNF-α increased in TNC1 astrocytes after OGD. (A) Western blot shows expression changes of TNF-α and GFAP protein in TNC1 astrocytes. (B) The quantitative analysis of TNF-α and GFAP. The results showed that the expressions of TNF-α and GFAP in OGD groups increased obviously. Significant differences are expressed as: *, P<0.05 *vs.* CON group. The values represent the mean ± SEM in triplicate. CON, control; OGD, oxygen-glucose deprivation; SEM, standard error of the mean; GFAP, glial fibrillary acidic protein; TNF-α, tumor necrosis factor-α.

above. After the end of hypoxia, the cell slides were taken out, and washed with PBS, 4% paraformaldehyde was used for fixation, and 10% goat serum was used for sealing. The slides were then placed in the primary antibody and incubated overnight at 4 ℃. The next day, the fluorescent secondary antibody Cy3-labeled goat against rabbit or goat against mouse (1:200) was incubated at room temperature, and the capsules were sealed with a fluorescent mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Sigma, art. No. F6057, USA). After the experiment, the images were collected using the positive fluorescence microscope, and the data were analyzed by Image J. All experiments were repeated at least three times.

### *Transwell migration assay*

Transwell migration array was used to evaluate the effect of GAS on the migration of TNC1 astrocytes. The TNC1 cell suspension was collected and inoculated into the upper layer of Transwell at a cell density of $1\times10^4$ cells/mL. After that, different conditional media (CON, GAS, 20% FBS) were added into the 24-well plate (600 μL/well). The GAS group was replaced with conditional medium (CM) after 1 h of treatment. After incubation in a cell incubator for 48 h, the medium was discarded and the remaining cells in the upper layer of Transwell were carefully removed. The lower layer cells were fixed with 100% methanol at room temperature for 15 min and then stained with 0.5% crystal violet. Images of migrating cells were captured ×200 magnification. Image J software was used to count the cells in the five visual fields on each insert. The results were expressed as mean ± standard error of the mean (SEM) of the number of cells per field of vision.

### *Statistical analysis*

All data were imported into Prism 8 (GraphPad Software, La Jolla, CA, USA) for statistical analyses. The data were presented as mean ± SEM. After the homogeneity test of variances, one-way analysis of variance (ANOVA) followed by multiple comparison of Dunnet's test was used to determine the statistical significance of the different groups. The difference was considered statistically significant when P<0.05.

## Results

### *Astrocytes activation increased after OGD*

Markers of reactive astrocyte, GFAP and TNF-α protein expression levels were detected after OGD in TNC1 cells. Compared with the Control group, the protein levels of GFAP and TNF-α were significantly upregulated (*Figure 1*), which suggested that TNC1 astrocytes were activated after OGD.

### *Detection of the effect of GAS on TNC1 cell viability*

Different concentrations of GAS had no significant toxic or lethal effects on the viability of TNC1 cells, and there was no statistically significant difference in cell mortality (P>0.05) (*Figure 2A*), our previous study found that GAS at 0.34 mM concentration had the most significant anti-inflammatory effect, therefore, 0.34 mM GAS was selected for follow-up study. Compared with untreated cells, the activity of

 

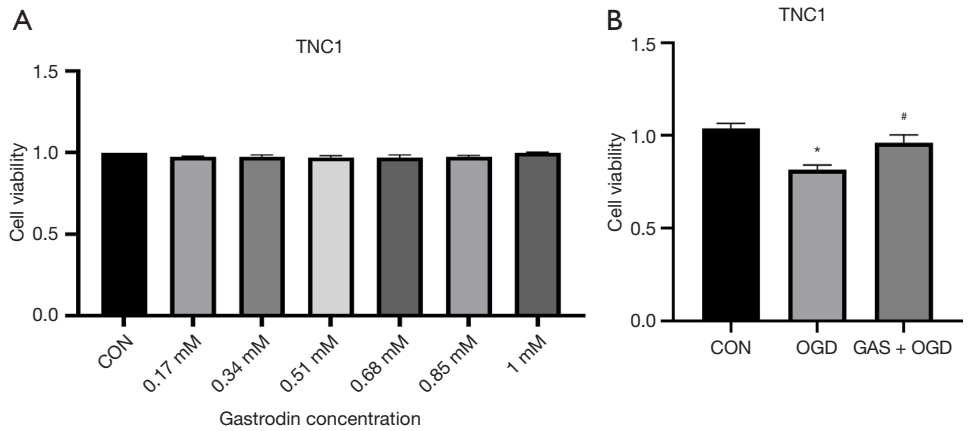


**Figure 2** The cell viability of TNC1 astrocytes cells. (A) The mortality of TNC1 astrocytes cells at different concentrations of GAS. Different concentrations of GAS had no significant toxic or lethal effects on the viability of TNC1 cells, and there was no statistically significant difference in cell mortality. (B) The cell viability of TNC1 astrocytes in different groups. The cell viability of OGD groups was lower than that of the CON group. The cell viability of groups with GAS increased obviously compared with OGD group, and the difference was statistically significant. Significant differences are expressed as: *, P<0.05 *vs.* CON group; #, P<0.05 *vs.* OGD group. The values represent the mean ± SEM in triplicate. CON, control; GAS, gastrodin; OGD, oxygen-glucose deprivation; SEM, standard error of the mean.

TNC1 cells was decreased after OGD treatment, and the difference was statistically significant (P<0.05) (*Figure 2B*). It is suggested that under the condition of low oxygen and no sugar, astrocytes are vulnerable to damage leading to cell death. After pretreatment with GAS for 1 h, the cell viability of the GAS + O group was significantly improved compared to the OGD group (P<0.05) (*Figure 2B*).

### *Exploring the effect of GAS on the migration function of TNC1 cells*

Compared with the CON group, the cell migration rate of the GAS-treated group was significantly increased, which was basically consistent with the effect of using the medium containing 20% FBS, suggesting that GAS also had a significant effect on the migration function of TNC1 cells (*Figure 3*).

### *Immunofluorescence staining to test the effects of GAS on expression of pro-inflammatory cytokines (TNF-α), neurotrophic factors (IGF-1 and BDNF), and Sirt3 in the OGD model of TNC1 cells*

To investigate the neuroprotective effect of GAS on TNC1 cells, the differential expression of pro-inflammatory cytokines (TNF-α), neurotrophic factors (IGF-1 and BDNF), and Sirt3 were identified using immunofluorescence staining (*Figure 4*). According to our results, the expression of TNF-α in TNC1 cells in the OGD group was significantly increased than that in the CON group, while the expression of TNF-α in the GAS + O group was significantly decreased than that in OGD group, indicating that GAS can significantly improve the pro-inflammatory environment caused by ischemia and hypoxia (*Figure 4A*).

The expression of neurotrophic factors showed that IGF-1 and BDNF expression in the OGD group were slightly increased, while their expression in the GAS + O group was markedly elevated than that in OGD groups (*Figure 4B,4C*). This suggested that GAS treatment can contribute to the secretion of neurotrophic factors.

For Sirt3 expression analysis, Sirt3 expression in the OGD group was notably upregulated than that in the CON group, while that in the GAS + O group was more obvious than that in OGD groups, further highlighting the potential mechanism of GAS's neuroprotective effect (*Figure 4D,4E*).

### *Western blotting detection for the effects of GAS on the expression of pro-inflammatory cytokines (TNF-α and IL-1β), neurotrophic factors (IGF-1 and BDNF), and Sirt3 in the OGD model of TNC1 cells*

The neuroprotective factors' expression of BDNF and

 

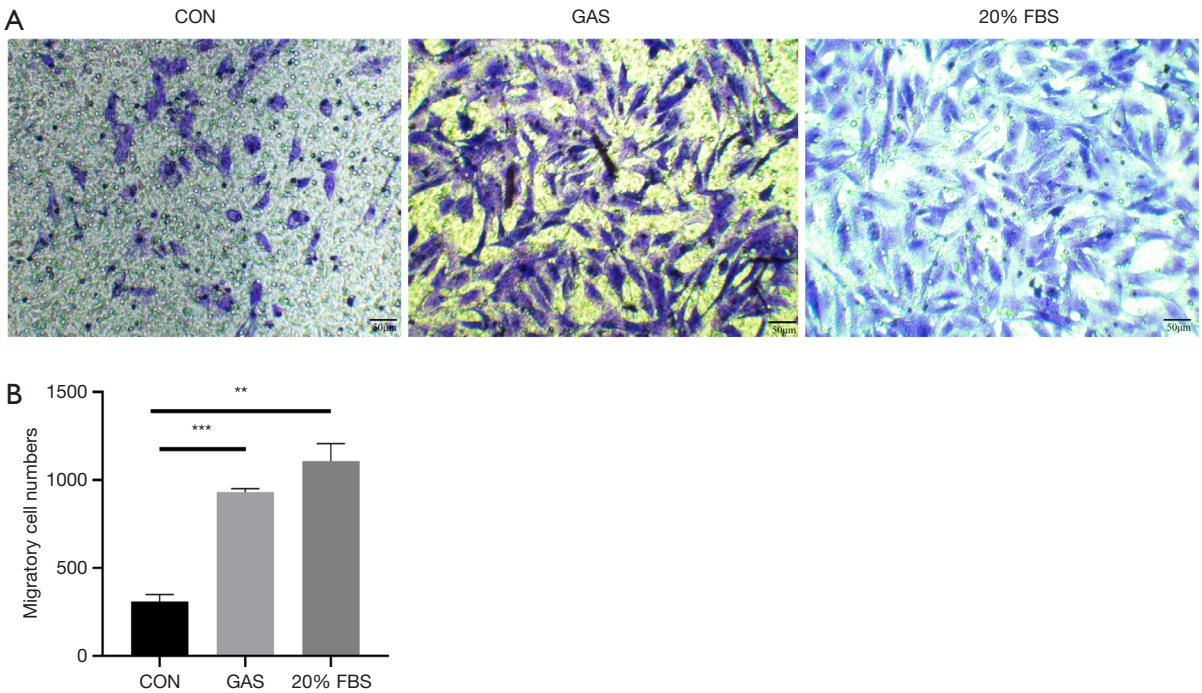


**Figure 3** GAS promoted the migratory ability of TNC1 astrocytes. (A) Light microscopy images show the number of positive TNC1 astrocytes in the GAS treated and 20% FBS groups (scale bars =50 μm, crystal violet staining). (B) The quantitative analysis reveals an increase in the migration of astrocytes with GAS treatment. Five random fields were counted for each group. Each experimental group was repeated three times. Data were presented as mean ± SEM. **, P<0.01; ***, P<0.001. CON, control; FBS, fetal bovine serum; GAS, gastrodin; SEM, standard error of the mean.

IGF-1 in the OGD group was increased than that in the CON group, although the difference has no statistically significance (*Figure 5A-5C*). GAS significantly increased the expression of BDNF and IGF-1 (*Figure 5B,5C*), which further verified that GAS treatment could contribute to the secretion of neurotrophic factors. The expression of Sirt3 was consistent with that of the neurotrophic factors (*Figure 5D*). The levels of IL-1β and TNF-α in the OGD group increased significantly compared to the CON group. However, GAS markedly reduced expression of IL-1β and TNF-α (*Figure 5E,5F*).

### *The effect of GAS on the expression of Notch signaling proteins in the OGD model of TNC1 cells*

With increasing evidence confirming that the Notch pathway is a key factor in the activation of astrocytes in the cerebral infarction area after stroke, as well as in the deterioration of brain injury and functional prognosis, we further investigated the effect of GAS treatment on the Notch signaling pathway. Both immunofluorescence (*Figure 6*) and Western blotting (*Figure 7*) results showed that Notch-1, NICD, Hes-1, and RBP-JK were significantly enhanced after OGD in TNC1 cells. However, pretreatment with 0.34 mM GAS markedly reduced the expression of these markers in reactive astrocytes. These results suggest that OGD can activate the Notch pathway and affect the function of TNC1 cells, GAS treatment can significantly inhibit this activation. We hypothesize that GAS therapy may act on inhibiting the Notch signaling pathway, which is worthy further study.

### *The Notch pathway inhibitor, DAPT, further verified the effect of GAS on Notch pathway protein expression*

We used the Notch pathway inhibitor, N-[N-(3,5-Difluorophenacetyl)-1-alany1]-S-phenyglycine t-butylester (DAPT), to further verify the effect of GAS on Notch pathway related protein expression (*Figure 8*). Our results showed that the expression of pathway proteins NICD and Hes-1 in TNC1 treated with DAPT decreased compared with that in the CON group. Compared with the OGD group, the expression of NICD, Hes-1, and RBP-JK were

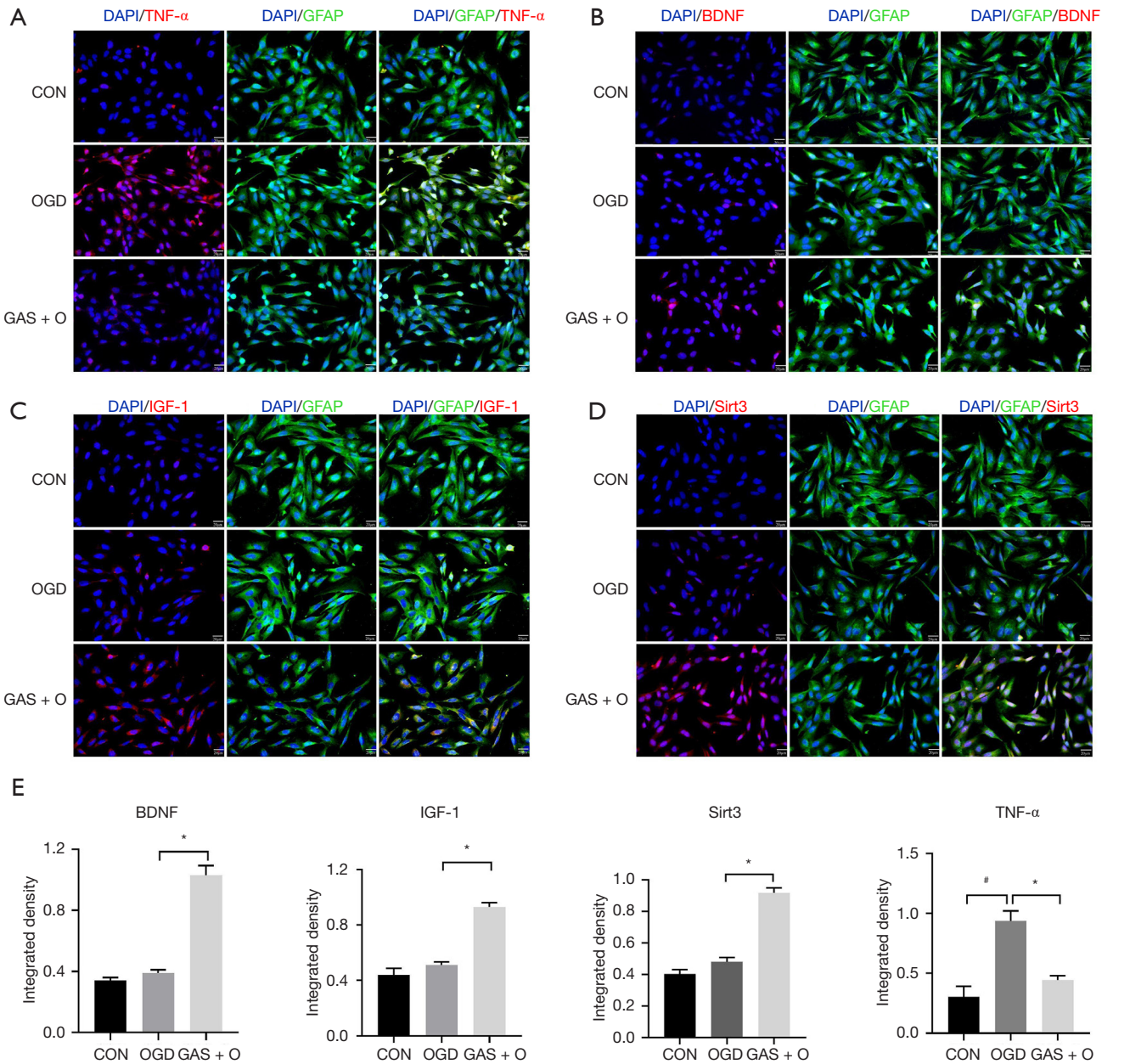


**Figure 4** GAS reduced TNF-α expression and enhanced BDNF, IGF-1, and Sirt3 expression in reactive astrocytes after OGD-stimulation. (A-D) Immunofluorescence images show TNF-α, BDNF, IGF-1, and Sirt3 (red) in GFAP-positive (green) astrocytes. DAPI (blue) shows the nuclei. (E) The quantitative analysis reveals BDNF, IGF-1, and Sirt3 expressions were drastically increased following treatment with GAS. TNF-α expression increased sharply in the OGD group, but were drastically decreased by GAS treatment. Significant differences are expressed as: #, P<0.05 *vs.* CON group; *, P<0.05 *vs.* OGD group. Scale bars: 20 μm. The values represented as mean ± SEM in triplicate. BDNF, brain-derived neurotrophic factor; CON, control; GAS, gastrodin; GFAP, glial fibrillary acidic protein; IGF-1, insulin-like growth factor-1; OGD, oxygen-glucose deprivation; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

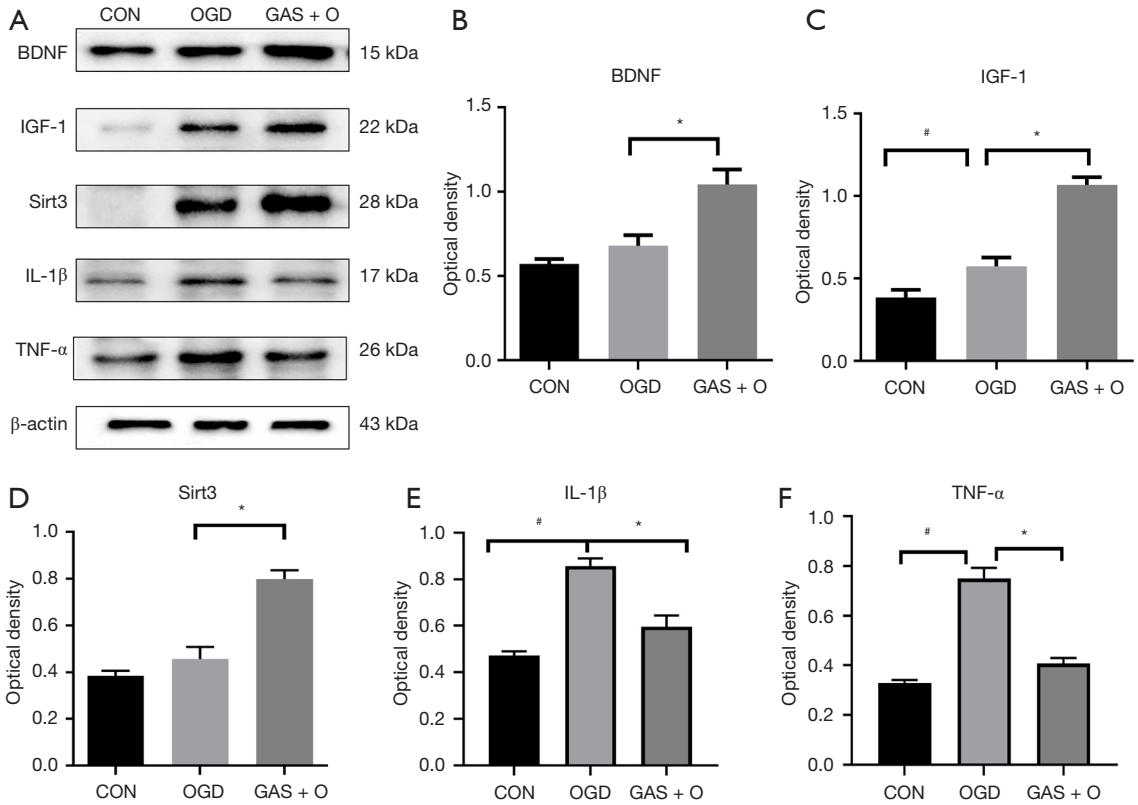


**Figure 5** GAS increased the protein expressions of BDNF, IGF-1, and Sirt3, but reduced the protein expressions of IL-1β and TNF-α in TNC1 astrocytes. (A) Western blotting results of BDNF, IGF-1, Sirt3, IL-1β and TNF-α. (B-F) The quantitative analysis of BDNF, IGF-1, Sirt3, IL-1β and TNF-α. The results showed that the expressions of BDNF, IGF-1, and Sirt3 in OGD groups had negligible increases, and increased obviously in groups with GAS. Meanwhile, IL-1β and TNF-α expressions were decreased in TNC1 astrocytes following treatment with GAS. Significant differences are expressed as: #, P<0.05 *vs.* CON group; *, P<0.05 *vs.* OGD group. The values represent the mean ± SEM in triplicate. BDNF, brain-derived neurotrophic factor; CON, control; GAS, gastrodin; IGF-1, insulin-like growth factor-1; IL-1β, interleukin 1β; OGD, oxygen-glucose deprivation; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

inhibited in the OGD + DAPT group, and their expression was further inhibited in the GAS+ OGD + DAPT group (*Figure 8A*,*8B*). Furthermore, the expression of Sirt3 in the DAPT group and OGD + DAPT group was also lower than that in the CON and OGD groups, respectively. Compared with GAS + O group, the expression of Sirt3 in combined treatment with DAPT and GAS group was decreased (*Figure 8C*,*8D*). Moreover, our results also showed that the combined application of DAPT and GAS further reduced the expression of TNF-α and IL-1β, and increased the expression of BDNF and IGF-1 (*Figure 8C*,*8D*).

## Discussion

In the early 1990s, Stevens *et al.* found that glial cells closely modulate and respond to neuronal activity and neurotransmission (27,28). Astrocytes are immune-active cells in the CNS. Current studies have shown that astrocytes express a wealth of receptors, ion channels, and second messenger systems, enabling them to influence the extracellular environment for survival, immune regulation, as well as signaling of nearby cells and neurons (29). Therefore, astrocytes play an important role in the physiological and pathological processes of the CNS, and may become a key therapeutic target of HIBD. In our study, after the intervention of GAS on reactive TNC1 astrocytes, it was found that GAS could significantly improve the effect of the hypoxic-sugar-free environment on TNC1 cell activity. In addition, GAS also significantly promoted the migration of TNC1 cells, suggesting that GAS improved the functional activity of astrocytes in HIBD.

Astrocyte overactivation-induced neuroinflammation

 

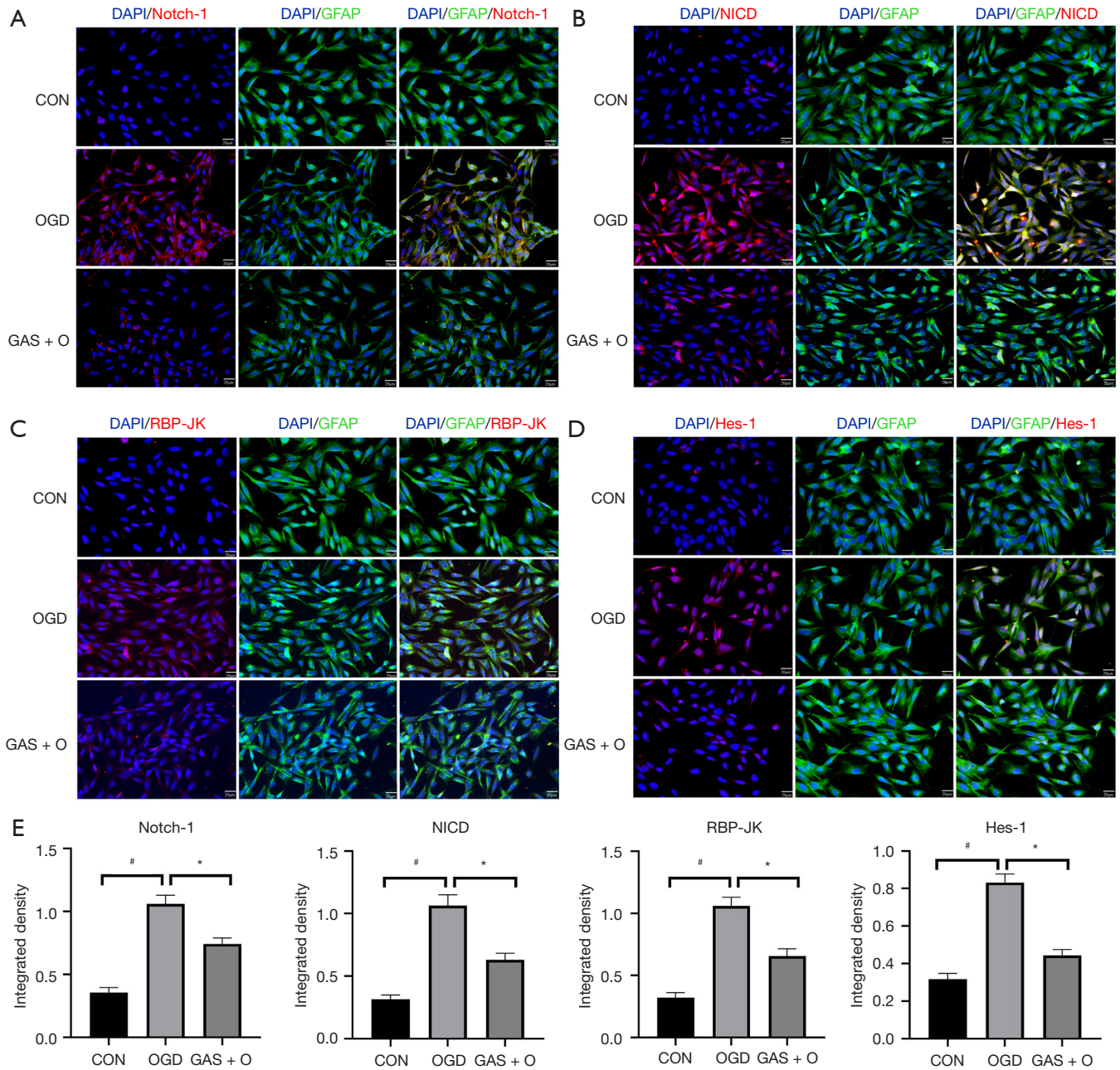


**Figure 6** GAS suppresses Notch-1, NICD, RBP-JK, and Hes-1 expression in OGD-activated TNC1 astrocytes. (A-D) Immunofluorescence images show that the expressions of Notch-1, NICD, RBP-JK, and Hes-1 (red) in GFAP-positive (green) astrocytes. (E) The quantitative analysis showed the expression of these proteins were increased in OGD group compared to the control, but were reduced following treatment with GAS. DAPI (blue) shows nuclei. Significant differences are expressed as: #, P<0.05 *vs.* CON group; *, P<0.05 *vs.* OGD group. Scale bars: 20 μm. The values represent the mean ± SD in triplicate. CON, control; GAS, gastrodin; GFAP, glial fibrillary acidic protein; Hes-1, transcription factor hairy and enhancer of split-1; NICD, intracellular Notch receptor domain; OGD, oxygen-glucose deprivation; RBP-JK, recombining binding protein suppressor of hairless; SD, standard deviation.

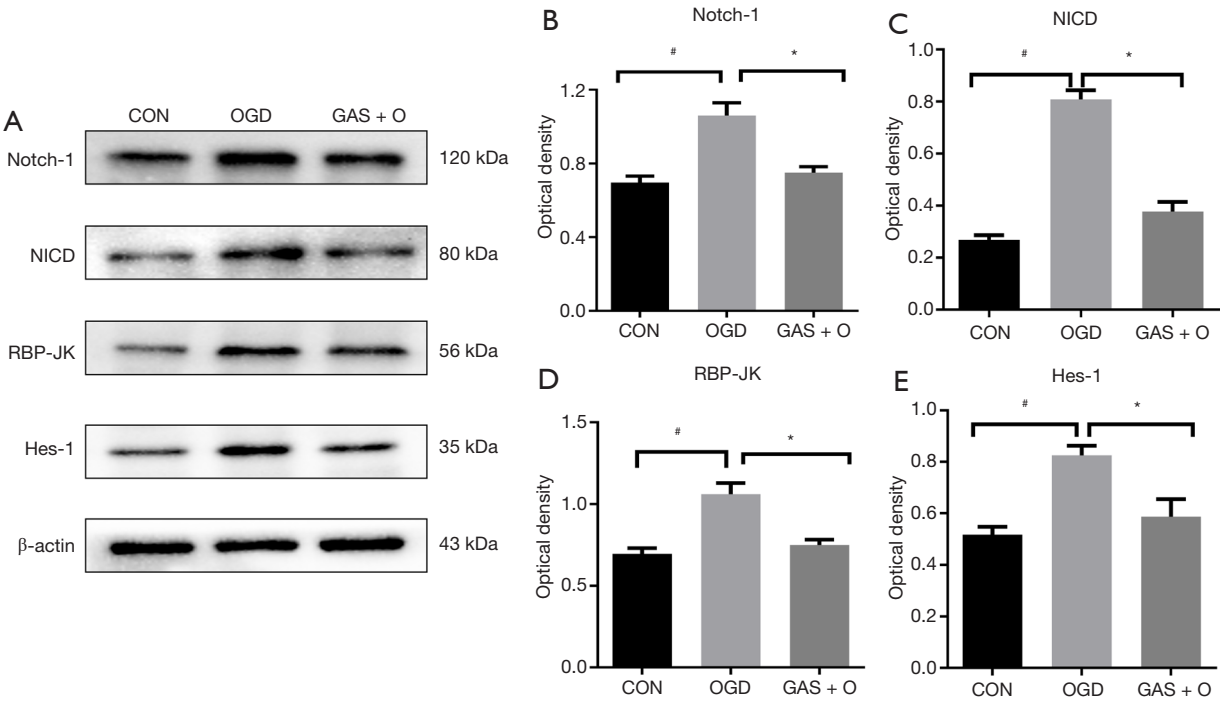


**Figure 7** GAS decreased the protein expressions of Notch-1, NICD, RBP-JK, and Hes-1 in TNC1 astrocytes after OGD. (A) Expression levels of Notch-1, NICD, and Hes-1 were significantly increased after OGD compared with the control. With GAS-treated TNC1, the expression levels of the above markers were further reduced compared with those of the OGD groups. (B-E) Bar graphs show the expression levels of Notch-1, NICD, RBP-JK, and Hes-1. Significant differences are expressed as: #, P<0.05 *vs.* CON group; *, P<0.05 *vs.* OGD group. The values represent the mean ± SEM in triplicate. CON, control; GAS, gastrodin; Hes-1, transcription factor hairy and enhancer of split-1; NICD, intracellular Notch receptor domain; OGD, oxygen-glucose deprivation; RBP-JK, recombining binding protein suppressor of hairless; SEM, standard error of the mean.

is a key feature of HIBD (30). Study has found that the occurrence of synchronous inflammatory responses in the brain can significantly promote HIBD-induced neuronal death (31). Previous studies have shown that early increases in pro-inflammatory cytokines, such as TNF-α, may contribute to a cascade of events causing delayed cellular death as well as subsequent tissue damage occurring in the hemisphere subjected to HIBD (32). Therefore, we cannot ignore the importance of hypoxia-induced neuroinflammatory responses. In our study, we found that GAS could downregulate the expression of inflammatory factors (IL-1β and TNF-α) in astrocytes after OGD model, which was in line with our expectations and previous studies (22). Hence, it is indicated that GAS can improve the neuroinflammatory response in HIBD.

Neurotrophic factors BDNF and IGF-1 play critical roles in neuronal survival, differentiation, synaptic plasticity, and maintenance of neurons following nerve injury (33). Meanwhile, study indicated that increased BDNF expression following stroke was not due to upregulated transcription, but rather increased uptake of the protein by astrocytes (34). When BDNF production in astrocytes is genetically depleted, neuronal damage and disease severity increase. Therefore, upregulation of BDNF and IGF-1 expression may have protective effect on injured neurons. In this study, we observed that GAS significantly increased the expression of BDNF and IGF-1 in TNC1 cells, which revealed that GAS could exert neuroprotective effects via upregulation of neurotrophic factors, BDNF/IGF-1 levels in reactive astrocytes may be critical for alleviating the neuroinflammatory response.

The sirtuin family is highly related with important metabolic regulatory pathways in prokaryotes and eukaryotes, and is involved in regulating various biological functions such as apoptosis, metabolism, stress responses, aging, differentiation and cell cycle progression (35). Sirt3,

 

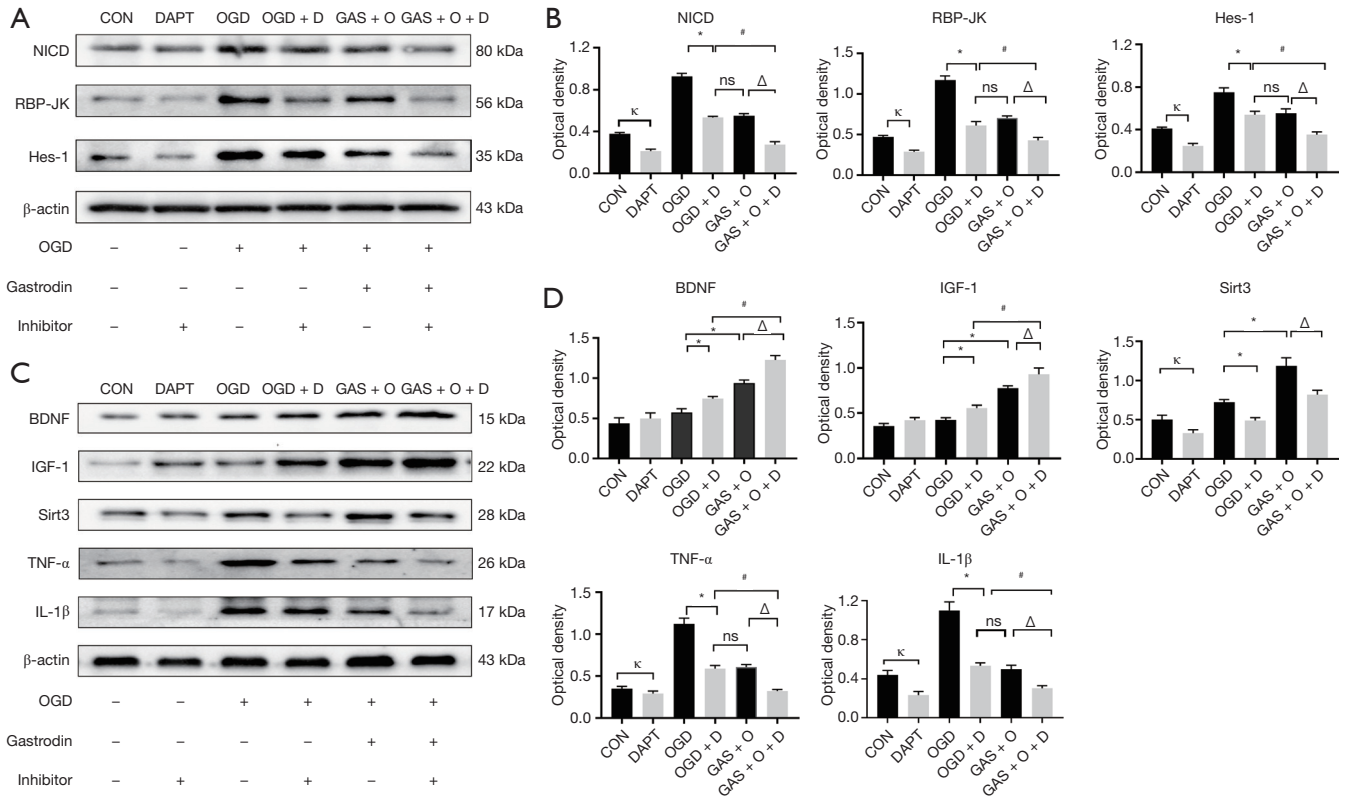


**Figure 8** DAPT further enhanced the effect of GAS on the expressions of Notch pathway proteins, neuroinflammatory factors, and neurotrophic factors. (A) Protein expression of NICD, RBP-JK and Hes-1. (B) The quantitative analysis showed the expression levels of NICD, RBP-JK, Hes-1 in OGD-activated TNC1 astrocytes were reduced significantly with DAPT treatment, in the GAS + OGD + DAPT (GAS + O + D) group, the expressions of NICD and HES-1 were further reduced. (C) Protein expression levels of BDNF, IGF-1, Sirt3, IL-1β, and TNF-α. (D) The quantitative analysis showed BDNF and IGF-1 level in OGD-activated TNC1 astrocytes were obviously increased with DAPT treatment. BDNF and IGF-1 expressions in OGD-activated TNC1 astrocytes administered a combined DAPT and GAS treatment reached a peak increase compared to the OGD + D or GAS + OGD (GAS + O) groups. Sirt3 expression in OGD-activated TNC1 astrocytes administered a combined DAPT and GAS treatment was reduced significantly compared with the OGD + D group. In OGD-activated TNC1 astrocytes, the IL-1β and TNF-α levels were reduced significantly with DAPT treatment. Combination of DAPT and GAS cumulatively decreased the expressions of IL-1β and TNF-α. Significant differences are expressed as: $^{\kappa}$, $P<0.05$ between the control and DAPT group; *, $P<0.05$ compared with the OGD group; $^{\#}$, $P<0.05$ between the OGD + D and GAS + O + D group; ns, $P>0.05$ compared with the OGD + D group; $^{\Delta}$, $P<0.05$ compared with the GAS+ O group. The values represent the mean ± SEM in triplicate. DAPT, N-[N-(3,5-Difluorophenacetyl)-1-alany1]-S-phenyglycine t-butylester; GAS, gastrodin; Hes-1, transcription factor hairy and enhancer of split-1; IGF-1, insulin-like growth factor-1; IL-1β, interleukin 1β; NICD, intracellular Notch receptor domain; ns, not significant; OGD, oxygen-glucose deprivation; RBP-JK, recombining binding protein suppressor of hairless; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α; ns, no significance.

one of seven members of the sirtuin family, is involved in the pathology of neurological diseases by regulating gene expression and enzyme activity related to oxidative metabolism and stress response (36). The present study demonstrated that the restoration of Sirt3 after brain injury (such as hemorrhage) reduced inflammation, mitochondrial damage, and oxidative stress, thereby exerting neuroprotective role (37). In addition, Sirt3 has been shown to exert its protective role in improving neuronal survival and sensorimotor function in ischemic stroke by regulating hypoxia induced factor 1α (HIF-1α) in astrocytes (38). Based on our data, GAS can significantly

promote the expression of Sirt3, which reveals the potential mechanism of GAS's neuroprotective effect. Given previous studies have suggested that Sirt3 may be related to the Notch signaling pathway (22,39), we further explored the role of Notch pathway in reactive astrocytes.

The Notch pathway is conserved as an evolutionarily critical signaling mechanism regulating the proliferation and differentiation of neural stem cells (40), and promoting the differentiation of astrocytes with hypoxia exposure (41,42). An increasing number of studies have shown that the Notch pathway is a key factor required for astrocyte activation in the infarct region, as well as worsening brain damage and functional outcomes after stroke (43). Recently, Acaz-Fonseca *et al.* (44) found that Notch signaling was involved in the morphological changes of reactive astrocytes subjected to inflammatory challenge. Following the application of the γ-secretase inhibitor, DAPT, all of the morphological, neurological, and biochemical changes were reversed. In this study, we found GAS significantly decreased the Notch signaling pathway upregulation induced by OGD, confirmed that neuroprotective effects of GAS can be exerted by modulating the Notch signaling pathway. However, the contributions of Notch signaling to the activation of astrocytes after in HIBD animals have not been explored.

According to our results, the expressions of TNF-α and the Notch pathways were markedly increased after OGD treatment, while GAS could significantly reduce the expression of these factors. When treated with Notch pathway inhibitor, DAPT, the expression of these factors was also significantly reduced, and the combined use of GAS and DAPT could make this reduction trend even more obvious. Notably, the combined use of GAS and DAPT could further reduce the expression of TNF-α and IL-1β, and increase the expression of BDNF and IGF-1. Interestingly, Sirt3 expression increased after OGD in TNC1 astrocytes, and decreased in the OGD + DAPT group. In addition, the expression of Sirt3 in OGD-stimulated TNC1 under DAPT combined with GAS treatment did not increase as expected. This shows that Sirt3 is a downstream gene of the Notch signaling pathway, which was consistent with our previous research (22). These results suggest that reactive astrocytes exert anti-inflammatory and secreting neurotrophic factors effects through regulating notch signaling pathway, which may be the target of GAS's protective effects on various nervous systems through astrocytes. However, other pathways should also be considered, which requires further research. The current results provide strong evidence supporting GAS as a promising therapeutic strategy for several neurological diseases, especially HIBD. This was worthy of further study.

## Conclusions

In conclusion, this study clearly showed that GAS can improve the cell activity and migration function of astrocytes after OGD, significantly inhibit the expression of pro-inflammatory factors (TNF-α and IL-1β), and markedly promote the expression of neurotrophic factors (BDNF and IGF-1). Furthermore, GAS could significantly enhance the expression of the Sirt3 protein and inhibit the Notch pathway. More importantly, the use of the Notch pathway inhibitor further confirmed that the neuroprotective effect of GAS is closely related to the regulation of Notch pathway activation.

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## Footnote

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appropriately investigated and resolved.



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