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Inhibition of GSK-3β alleviates cerebral ischemia/reperfusion injury in rats by suppressing NLRP3 inflammasome activation through autophagy

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ABSTRACT

The nod-like receptor protein 3 (NLRP3) inflammasome has a critical role in cerebral ischemic injury, and autophagy is related to activation of the inflammasome under oxidative stress conditions. However, it is unclear how NLRP3 inflammasome activation is regulated. Glycogen synthase kinase 3β (GSK-3β) emerged as an important risk factor for brain ischemia reperfusion injury, and GSK-3β inhibits autophagic activity in many diseases. In this study, we examined whether NLRP3 inflammasome-derived inflammation could be ameliorated by GSK-3β inhibition in a cerebral ischemia reperfusion injury model and assessed whether autophagy is involved in this process. To establish ischemic reperfusion injury, we used a middle cerebral artery occlusion-reperfusion (MCAO/R) model in rats. A chemical inhibitor (SB216763) and GSK-3β siRNA were used to suppress GSK-3β activation and GSK-3β expression in vivo. The results demonstrated that SB216763 and GSK-3β siRNA improved neurological scores, reduced cerebral infarct volume, and decreased the levels of NLRP3 inflammasome, cleaved-caspase-1, IL-1β, and IL-18. Inhibiting GSK-3β activation enhanced autophagic activity (ratio of LC3B-II/LC3B-I and p62/SQSTM1), whereas treating with an autophagy inhibitor (3-MA) abrogated the inhibitory effect on NLRP3 inflammasome activation after GSK-3β inhibition. These results suggest that inhibiting GSK-3β downregulates NLRP3 inflammasome expression by increasing autophagic activity in cerebral ischemia reperfusion injury. GSK-3β might be an attractive specific target and that it functions by regulating the NLRP3 inflammasome.

1. Introduction

Ischemic stroke is a major cause of destructive cerebrovascular disease and can have harmful effects on human health. It has high morbidity, high incidence, and high mortality [1]. Cerebral ischemia and reperfusion (I/R) occur following stroke [2]. The currently available clinically effective therapies for I/R have several limitations, largely because of the complexity of the pathophysiological mechanisms underlying the injury [1]. Previous studies provided increasing amounts of evidence showing that the inflammasome plays a key role in I/R injury [3,4].

NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasomes are multi-proteins that aggregate upon the intracellular presence of damage- and pathogen-associated molecular patterns (DAMPs/PAMPs) and typically consist of the receptor protein NLRP3, the adaptor protein PYCARD/ASC, and the protease CASP1/caspase-1 [3,5]. Once assembled and activated, the inflammasome prompts the cleavage of pro-CASP1 into its active form. Active CASP1 then cleaves the precursors of several inflammatory molecules, including converting IL-1β (interleukin-1β) and IL-18 (interleukin-18) into their mature forms, thus aggravating the inflammatory reaction [5,6,41]. Increasing amounts of evidence have suggested that the NLRP3 inflammasome is activated in several inflammatory diseases such as Alzheimer's disease [7], type II diabetes mellitus [8], atherosclerosis [9], and cancers [10].

Abbreviations: NLRP3, nod-like receptor protein 3; GSK-3β, glycogen synthase kinase 3β; MCAO/R, middle cerebral artery occlusion-reperfusion; I/R, ischemia and reperfusion; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; IL1β, interleukin-1β; IL-18, interleukin-18; LC3, microtubule-associated protein-1 light chain-3; 3-MA, 3-methyladenine; DMSO, dimethyl sulfoxide; TTC, triphenyltetrazolium chloride; CBF, local cerebral blood flow

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Recent research demonstrated that the NLRP3 inflammasome plays a vital role in the development of ischemia/reperfusion-induced brain injury [11,12]. Thus, controlling NLRP3 inflammasome activation is meaningful and of particular importance.

GSK3β is a serine/threonine kinase that participates in signaling pathways via phosphorylation-mediated signal cascades [20,21]. GSK-3β inactivation was suggested to promote neuronal survival [22]. Inhibiting GSK-3 kinase activity by transfection with dominant-negative forms of GSK-3β partially protected cortical neurons from glutamate-induced excitotoxicity, whereas overexpression of wild-type GSK-3β induced neurotoxicity in the absence of glutamate and potentiated the toxicity in the presence of glutamate [23]. GSK-3β activation requires phosphorylation at tyrosine 216 [18,21]. In the midbrain and pons, the activated (pY216) form of GSK-3β may be involved in the paraquat-induced degeneration processes progressing in catecholaminergic neurons [40]. Together, these reports suggest that GSK-3β has multifunctional roles. Moreover, previous studies revealed that GSK-3β inhibits autophagy [18,19].

Autophagy is an evolutionarily conserved cellular process that addresses the problems of misfolded or aggregated proteins, damaged organelle disposal, and invasion by pathogens. It acts via the a lysosomal degradation pathway for clearance [13–15]. Active autophagy reduces inflammation by directly eliminating active inflammasomes and indirectly removing inflammatory stimuli such as reactive oxygen species (ROS), intracellular pathogenic microorganisms, or damaged organelles to maintain cellular homeostasis [15–17]. Previous observations suggested that autophagic activity can inhibit the activation of NLRP3 inflammasomes and IL-1β expression by eliminating mitochondrial DNA (mtDNA) and mitochondrial ROS (mtROS) in brain I/R injury [17].

In this study, middle cerebral artery occlusion-reperfusion (MCAO/R) was used to establish I/R injury and the inhibitor SB216763 and GSK-3β siRNA were used to suppress GSK-3β. We hypothesized that inhibiting GSK-3β reduces activation of the NLRP3 inflammasome by increasing autophagy.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University [24]. Male Sprague-Dawley rats (aged 60–80 d, 240–280 g) were procured from the Chongqing Medical Animal Experimentation Center. A total of 200 adult rats were used. All rats were identified by earmarks and numbered accordingly. Using a table of random numbers animals were randomly divided into 7 groups. Animals from experimental group were always treated first and then divided randomly into Sham group (sham-operated), MCAO group(Transient cerebral ischemia and reperfusion), siRNA group(GSK-3βsiRNA injected before MCAO), ConsiRNA group (scramble siRNA injected before MCAO), SB216763 group(SB216763 injected before MCAO), 3-MA group(3-Methyladenine injected before MCAO) and SB216763 + 3-MA group(SB216763 and 3-MA injected before MCAO). All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the animals' suffering.

2.2. Reagents and antibodies

3-Methyladenine (3-MA), SB216763, dimethyl sulfoxide (DMSO), and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies used were as follows: GSK-3β (Cell Signaling Technology #12456), GSK-3β (phospho Y216; Abcam, ab75745), NLRP3 inflammasome (Santa Cruz Biotechnology, CA, US, sc-66,846), caspase-1 (Abcam, ab108362), IL-1β(Affinity Biosciences, Asp116), IL-18 (R&D systems, MAB521-SP), β-actin (ABclonal, Wuhan, China, AC004), LC3B (Proteintech, US, 18725–1-AP), and p62/SQSTM1 (Proteintech, 18,420–1-AP). The secondary antibodies included HRP-conjugated anti-rabbit IgG (H + L) (ABclonal, AS014) and HRP-conjugated anti-mouse IgG (H + L) (ABclonal, AS003).

2.3. MCAO model

Male Sprague-Dawley rats (240–280 g) received food and water freely before the operation. They were randomly divided into 7 groups: Sham group, MCAO group, siRNA group, ConsiRNA group, SB216763 group, 3-MA group and SB216763 + 3-MA group. The MCAO model was described in our previous studies [3,25]. Rats were deeply anesthetized with 3.5% chloral hydrate by intraperitoneal injection (350 mg/kg) and then subjected to the operation on a heating pad to maintain core temperature. A nylon filament (Beijing Cinontech Co. Ltd., Beijing, China) with a rounded tip was placed inside the left middle cerebral artery for 60 min. The nylon filament was inserted until it reached the origin of the middle cerebral artery (MCA) and local cerebral blood flow (CBF) decreased to < 16% of the baseline level. Then, the nylon filament was slowly taken out from the ischemic artery to restore blood flow, which initiated reperfusion. Rats in the sham group underwent the same operation but without MCAO. Rats that died or in which blood reperfusion remained beneath 70% were removed from the study.

2.4. Preparation and injection of siRNA, 3-MA, and SB216763

siRNA against GSK-3β (sense, 5′-GGAGAGCCCAAUGUUUCAUTT-3′; and antisense, 5´-AUGAAACAUUGGGCUCUCCTT-3′) was designed and synthesized by GenePharma Corporation (Shanghai, China). A scrambled siRNA (sense, 5´-GCGCCAGUGGUACUUAAUATT-3′; antisense, 5′-UAUUAAGUACCACUGGCGCTT-3′) without a target sequence was synthesized as a control. 3-MA was stored at room temperature and dissolved in DMSO (10 mg/ml) with gentle heating to yield a clear, colorless solution before injection. SB216763 was stored at −20 °C, dissolved inDMSO (20 mg/ml) and diluted with saline. Rats were deeply anesthetized and then placed on a stereotaxic apparatus (Taimeng Software, Chengdu, China). A 25-μL Hamilton syringe was fixed on the stereotaxic apparatus and inserted perpendicularly at 1.0 mm posterior to bregma, 2.0 mm lateral to midline, and to a depth of 3.5 mm beneath the surface of the skull. Next, 15 μL of GSK-3β siRNA (2 μg/μL) was injected into the left lateral ventricle at a rate of 1 μL/min according to the manufacturer's instructions. The needle was kept in place for 15 min after the injection was complete and then withdrawn slowly. GSK-3βsiRNAs were injected into the left lateral cerebral ventricle 24 h before MCAO. SB216763 (20 μg/kg) was injected into the left lateral ventricles 6 h before MCAO and 3-MA (600 nmol,10 μg/μL) was injected into the left lateral ventricles 30 min before MCAO.

2.5. Measurement of infarct volume

After 24 h of reperfusion, the animals were anesthetized by chloral hydrate injection (400 mg/kg, i.p.) decapitated, and the whole brains were quickly removed and frozen at −20 °C for 15 min. Brain tissues were cut into five coronal sections (2 mm thick), stained with 2% TTC at 37 °C for 15 min, and fixed in 4% paraformaldehyde at 4 °C for 48 h. The sections were photographed with a digital camera and analyzed using ImageJ analysis software (version 6.0, NIH). The percentage of the area that was infarcted was calculated using the following equation: (right volume − (left volume − left infarct volume)/right volume × 100%.

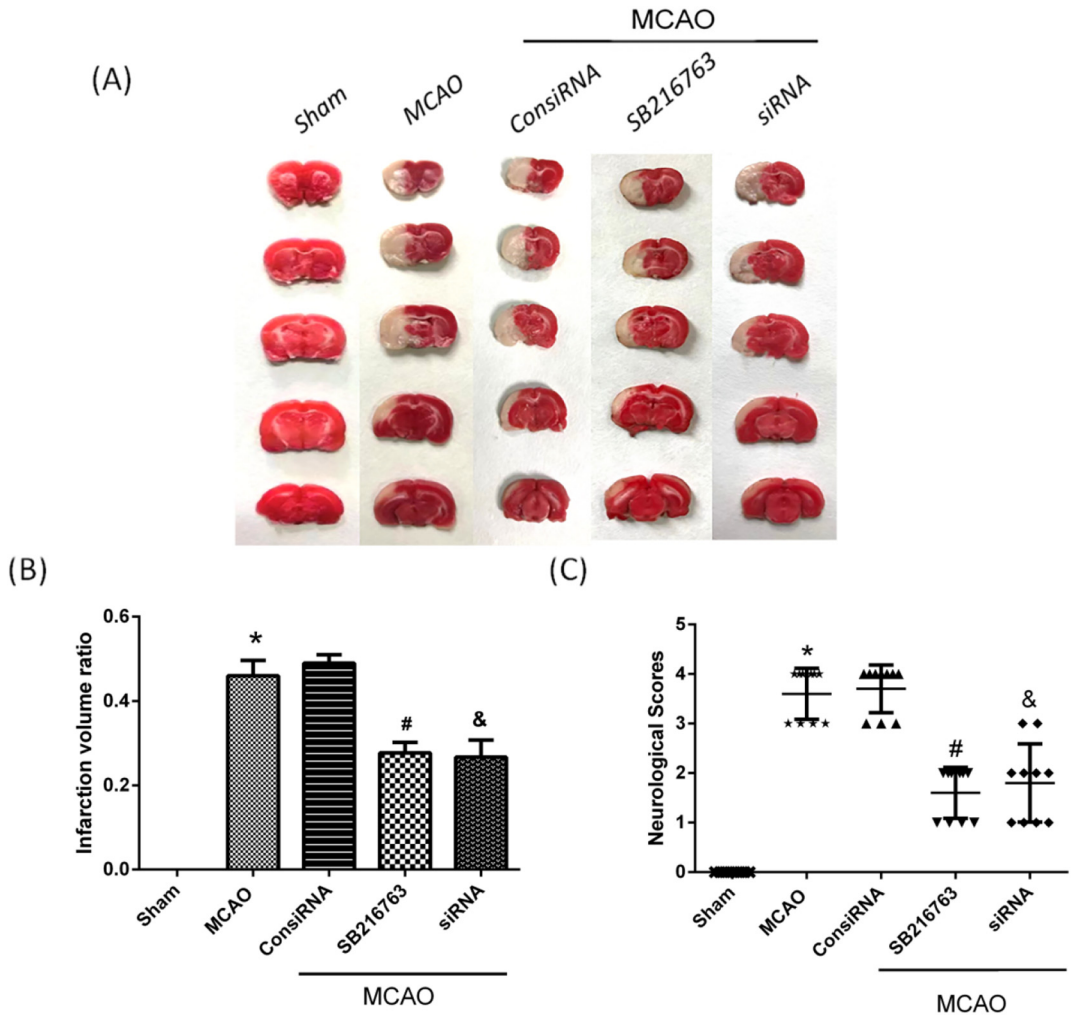

Fig. 1. Effects of SB216763 treatment and GSK-3β knockdown on cerebral infarct volume and neurological scores. (A, B) Cerebral infarct volume was reduced after SB216763 treatment and GSK-3βknockdown compared with the MCAO group. (C) Neurological scores were improved after SB216763 treatment and GSK-3βknockdown compared with the MCAO group and ConsiRNA group. All the data are represented as means ± SD. (MCAO vs. Sham,*$P < 0.05$; MCAO + SB216763 vs. MCAO, #$P < 0.05$; MCAO + control siRNA vs. MCAO, &$P < 0.05$). $n = 10$ per group.

2.6. Measurement of IL-18 and IL-1βlevels

The animals were anesthetized by chloral hydrate injection after 24 h of reperfusion. The brains were quickly removed after saline perfusion and all proteins were collected from the same position of the ischemic side of the cerebral cortex. The cortices were homogenized in RIPA buffer containing the protease inhibitor PMSF. The mixed lysates were centrifuged at 12,000 ×*g* for 10 min at 4 °C, and the supernatants were collected. Protein concentrations were determined using the Sensitive BCA Protein Assay Kit (Beyotime, Jiangsu, China).IL-18 and IL-1β levels in the cerebral cortex were determined using ELISA kits (Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions and quantified using a microplate reader at 450 nm.

2.7. Electron microscopy

After saline perfusion, the brains were quickly removed. Them, three 1 mm tissue samples were collected from the ischemic side of the cerebral cortex and placed in 4% glutaraldehyde fixed solution for storage. All samples were examined under an electron microscope. The number of autophagosomes in neurons was determined by electron microscopy.

2.8. Measurement of neurologic deficit scores

Neurologic deficit scores were estimated at 24 h of reperfusion after MCAO using a modified scoring system reported previously [26] as follows: grade 0, normal (no apparent neurological deficits); grade 1, failure to fully extend the contralateral forelimb; grade 2, continuous circling to the contralateral side but standard posture at rest; grade 3, falling to the injured side; grade 4, no spontaneous autonomic activity and a sluggish level of consciousness; grade 5, death. Rats with grades of 0 and 5 were removed from the study.

2.9. Western blotting

The detailed process for the tissue disruption has been mentioned previously. Equal amounts of protein samples (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Millipore, Boston, MA, US). The membranes were blocked with 5% non-fat milk in TBST. All membranes were incubated with the appropriate primary antibody at 4 °C overnight followed by HRP-conjugated secondary antibody at room temperature for 2 h. Specific protein signals were detected using a chemiluminescence system. Each immunoblotting experiment was performed three times.

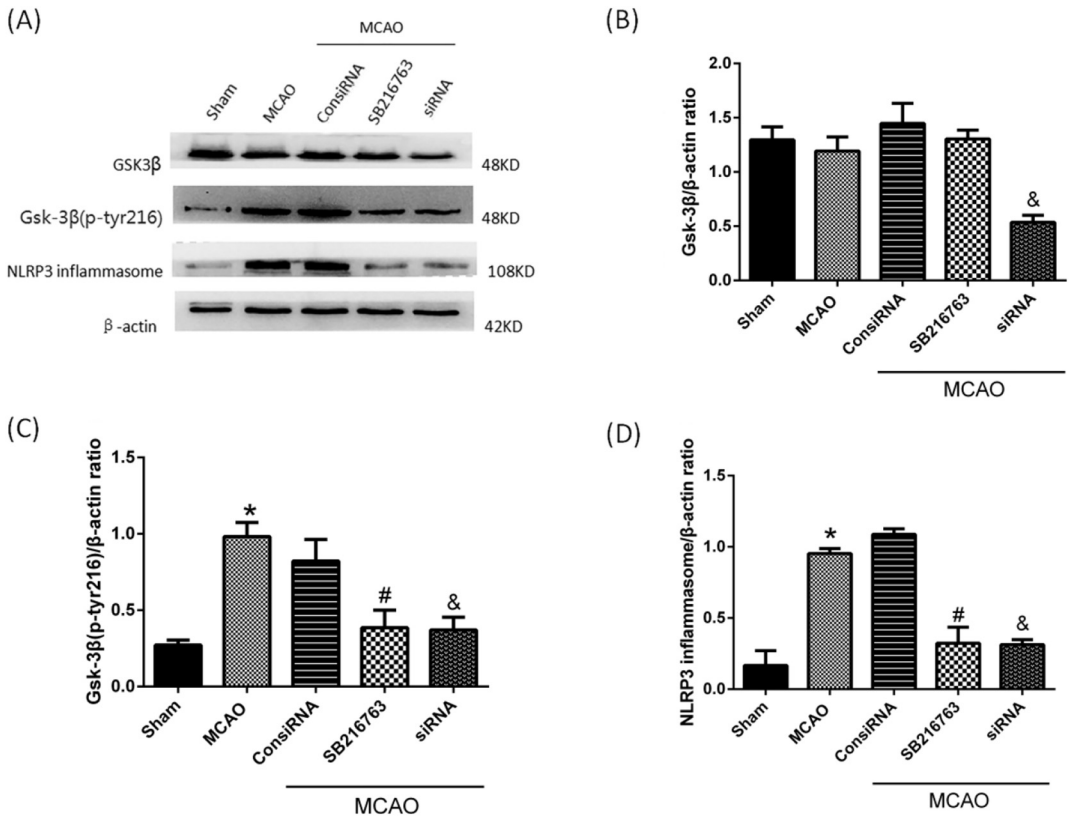

Fig. 2. SB216763 treatment and GSK-3β knockdown inhibits the NLRP3 inflammasome in cerebral MCAO/R injury in rats. (A) Representative western blots showing protein levels of GSK-3β, GSK-3β (p-Tyr216), and NLRP3 inflammasome; expression was normalized to β-actin. Quantification of the protein levels in (A) is shown in (B–D). All the data are represented as means ± SD. (MCAO vs. Sham, *$P < 0.05$; MCAO + SB216763 vs. MCAO, #$P < 0.05$; MCAO + control siRNA vs. MCAO, & $P < 0.05$). $n = 6$ per group.

2.10. Statistical analysis

Data are expressed as means ± SD. All statistical analyses were performed using GraphPad Prism software (version 6.0). Statistical differences among multiple groups were determined using one-way analysis of variance (ANOVA) followed by Student's-Newman-Keuls tests for multiple comparisons. Statistical significance was established when $P < 0.05$.Data points outside the 95% confidence interval were treated as outliers and excluded from the data analysis.

3. Results

3.1. GSK-3β siRNA and inhibitor alleviate cerebral I/R injury in rats

Since we previously reported that the expression of p-GSK-3β (Tyr216) remains increased in the 24-h following reperfusion after MCAO [21], we first assessed whether suppressing GSK-3β activity affects neurological function 24 h after MCAO. To investigate the protective effects of GSK-3β inhibition against cerebral I/R injury, we examined the effects of the inhibitor SB216763 and GSK-3β siRNA on cerebral infarct volume and neurological scores. The infarct volumes in the MCAO + SB216763 and MCAO + GSK-3β siRNA groups were significantly lower than in the MCAO group, and there was no significant difference in cerebral infarct volume between the MCAO and MCAO + control siRNA groups (Fig. 1A, B). The neurological function scores of rats in the MCAO group were significantly higher than those in the sham group, and SB216763 and GSK-3β siRNA remarkably improved neurological scores (Fig. 1C). These results suggest that SB216763 and GSK-3β siRNA significantly decreased I/R injury and improved neurological function.

3.2. Inhibiting GSK-3β inhibits NLRP3 inflammasome expression after MCAO

We previously demonstrated that the NLRP3 inflammasome plays a critical role in I/R injury and peaks ~24 h after MCAO [25]. To analyze whether suppressing GSK-3β activity affects NLRP3 inflammasome expression, we measured NLRP3 inflammasome levels 24 h after MCAO following treatment with SB216763 and GSK-3β siRNA. Western blotting revealed that the expression of p-GSK-3β (Tyr216) were significantly higher in the MCAO and MCAO + control siRNA groups than in the sham group (Fig. 2A,C). Total GSK-3β expression was lower in the GSK-3β siRNA group than the MCAO group (Fig. 2A, B). The expression of p-GSK-3β (Tyr216) was lower in the SB216763 and GSK-3β siRNA groups than the MCAO group (Fig. 2A, C). These observations indicate that SB216763 suppresses the activation of GSK-3β and GSK-3β siRNA inhibits both the activation and expression of GSK-3β.To assess the effect of GSK-3β inhibition on the NLRP3 inflammasome, the protein levels of activated NLRP3 inflammasome were measured using western blotting. As shown in Fig. 2A, D, I/R significantly elevated NLRP3 inflammasome levels, which was abrogated by treatment with SB216763 or GSK-3β siRNA. These findings suggest that GSK-3β inhibition may ameliorate I/R-induced brain injury by inhibiting the NLRP3 inflammasome.

3.3. Inhibiting GSK-3β inhibits cleavage of caspase-1, IL-1β, and IL-18 after MCAO

Since the assembly and activation of NLRP3 inflammasome leads to caspase-1 activation and the maturation of pro-IL-1β and pro-IL-18 into IL-1β and IL-18, respectively [3], we next explored whether suppressing GSK-3β activity affects effectors downstream of the NLRP3

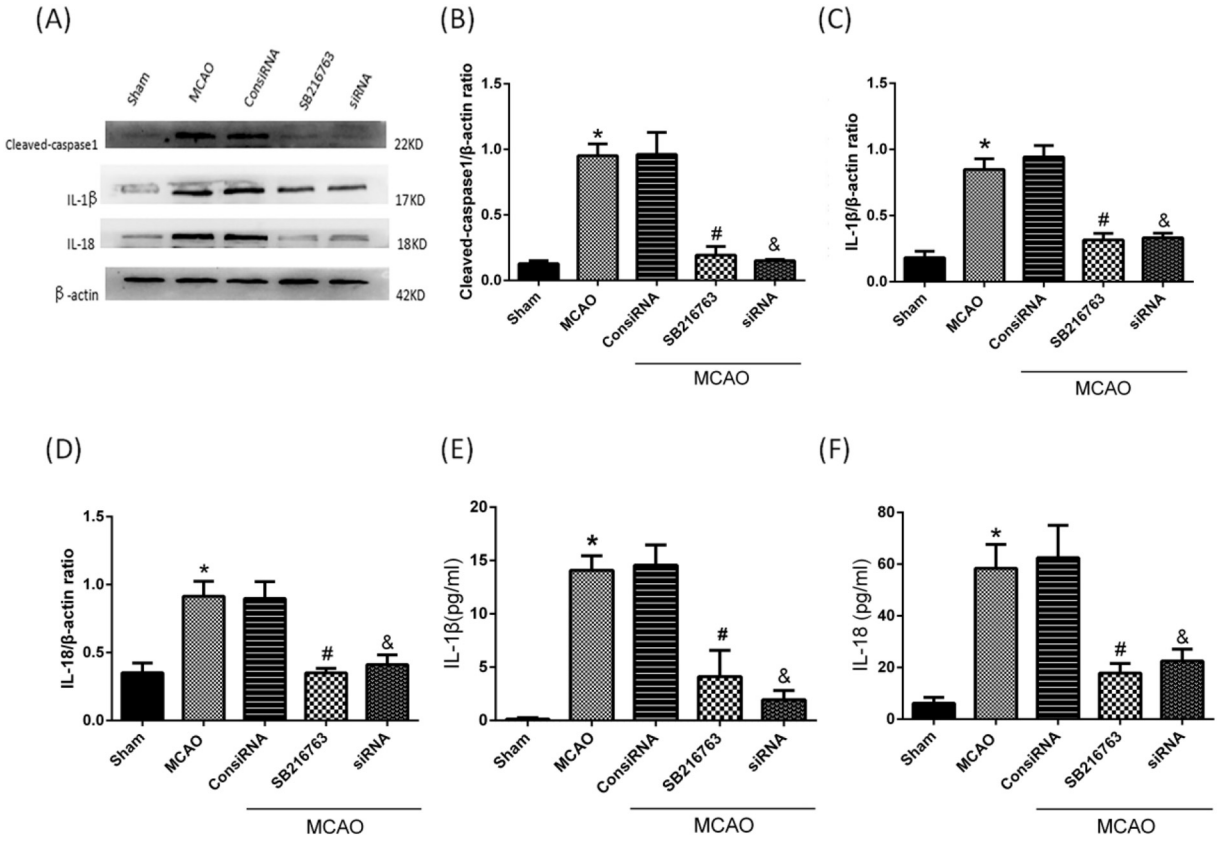

Fig. 3. SB216763 treatment and GSK-3β knockdown inhibit NLRP3 inflammasome-derived inflammation in cerebral MCAO/R injury in rats. (A) Representative western blots showing protein levels of cleaved-caspase-1, IL-1β, and IL-18; expression was normalized to β-actin. Quantification of the protein levels in (A) is shown in (B–D). IL-1β (E) and IL-18 (F) levels were measured using ELISA. All the data are represented as means ± SD. (MCAO vs. Sham,*$P < 0.05$; MCAO + SB216763 vs. MCAO, #$P < 0.05$; MCAO + control siRNA vs. MCAO, &$P < 0.05$). $n = 6$ per group.

inflammasome. To determine the effects of GSK-3β inhibition on the NLRP3 inflammasome-related release of cytokines, the protein levels of cleaved-caspase-1, IL-1β, and IL-18 were measured by western blotting. As shown in Fig. 3A–D, I/R significantly increased the levels of cleaved-caspase-1, IL-1β, and IL-18; these increases were abrogated by treatment with GSK-3β siRNA or SB216763. These findings suggest that inhibiting GSK-3β may alleviate I/R-induced brain injury by inhibiting the NLRP3 inflammasome-mediated release of cytokines.

3.4. Inhibiting GSK-3β augments autophagic activity in cerebral I/R injury

Our previous study revealed that increased GSK-3β activity suppresses autophagy in acute liver failure [38] and that autophagic activity can inhibit the activation of NLRP3 inflammasomes in brain I/R injury [17]. Therefore, we next tested whether the effects of GSK-3β on NLRP3 inflammasome activation are mediated by autophagy in brain I/R injury. We first analyzed whether suppressing GSK-3β activity enhances autophagy. To investigate the effect of inhibiting GSK-3β on autophagic activity 24 h after MCAO, we assessed the ratio of LC3B-II/LC3B-I and p62 expression using western blotting. The ratio of LC3B-II/LC3B-I and p62 levels were increased 24 h after MCAO. Treatment with GSK-3β siRNA and SB216763 increased the LC3B-II/LC3B-I ratio in the MCAO group and simultaneously decreased p62 levels (Fig. 4A–C). We also examined autophagosomes in neurons by electron microscopy. Compared with the MCAO group, there were more autophagosomes in the SB216763 and GSK-3β siRNA groups (Fig. 4D, E). These results suggest that inhibiting GSK-3β can enhance autophagic activity under I/R stimulation.

3.5. Inhibiting GSK-3β affects NLRP3 inflammasome activation and downstream effectors via autophagy

To further test the hypothesis that the effects of inhibiting GSK-3β on the regulation of NLRP3 inflammasome activation are mediated by autophagy, an autophagy inhibitor (3-MA) was used to suppress autophagic activity after treatment with SB216763 in MCAO. Although there was no significant difference in p-GSK-3β (Tyr216) levels between the MCAO and 3-MA groups and the decrease in p-GSK-3β (Tyr216) levels in SB216763 groups were not abrogated in SB216763 + 3-MA groups, levels of NLRP3 inflammasome, cleaved-caspase-1, IL-1β, and IL-18 were increased in the 3-MA groups compared with the MCAO groups. Pretreatment with 3-MA reduced the increase in autophagy compared with the SB216763 + MCAO (Fig. 5A, C, D) and MCAO groups by inhibiting GSK-3β and increasing the levels of NLRP3 inflammasome activation, cleaved-caspase-1, IL-1β, and IL-18 (Fig. 5A, E–H). In conclusion, these results suggest that GSK-3β inhibition suppresses NLRP3 inflammasome activation and cleaved-caspase-1, IL-1β, and IL-18 levels via autophagy.

4. Discussion

The present study showed that the NLRP3 inflammasome could be suppressed and thereby exert neuroprotective effects against cerebral MCAO/R injury. These effects were elucidated by inhibiting and knocking down GSK-3β, which revealed the relationship between the NLRP3 inflammasome and GSK-3β in the cerebral cortex of rats that sustained MCAO/R. Inhibiting or knocking down GSK-3β using siRNA reduced the infarct volume and improved the neurological behavior relative to the MCAO group. SiRNA knockdown and inhibition of GSK-

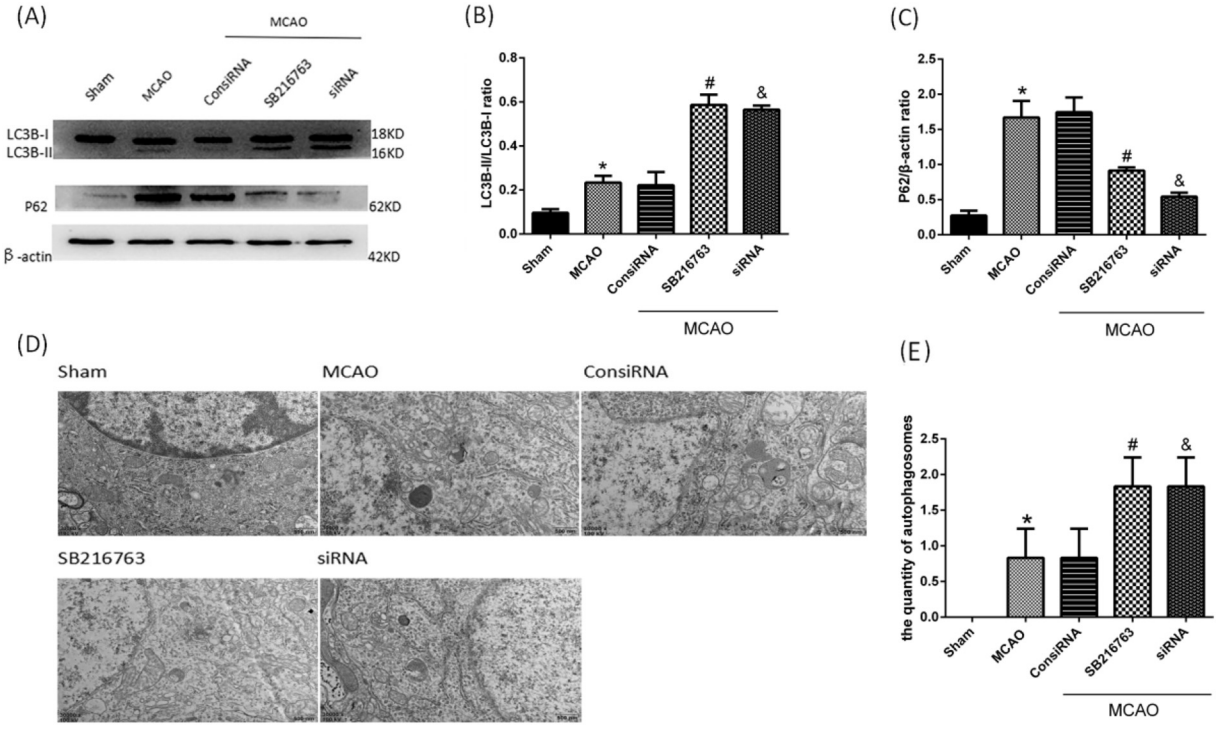

Fig. 4. SB216763 treatment and GSK-3β knockdown increased autophagic activity in cerebral MCAO/R injury in rats. (A–E) Autophagy activity was assessed by determining the ratio of LC3B-II/I, p62 levels, and the quantity of autophagosomes. Protein levels of p62 were normalized to β-actin. Quantification of the protein levels in (A) are shown in (B, C). The quantity of autophagosomes (red arrows) in neurons was determined by electron microscopy. (D, E) All the data are represented as means ± SD. (MCAO vs. Sham,*$P < 0.05$; MCAO + SB216763 vs. MCAO, #$P < 0.05$; MCAO + control siRNA vs. MCAO, &$P < 0.05$). $n = 6$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

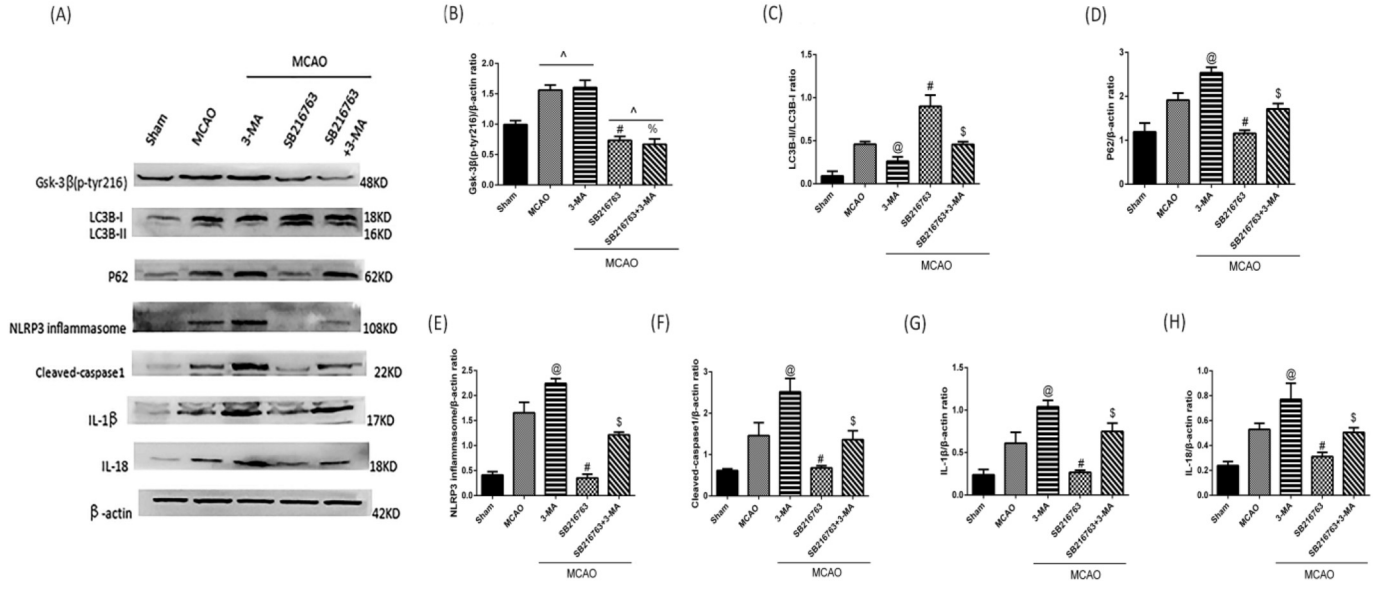

Fig. 5. 3-MA significantly reversed the effects of SB216763 treatment on autophagic activity and inflammasome-derived inflammation in cerebral MCAO/R injury in rats. (A, B) Pretreatment with 3-MA induced no significant change on GSK-3β (p-Tyr216) expression.(A, C, D)3-MA decreased the ratio of LC3B-II/LC3B-I and increased p62 levels. (A, *E*-H)Pretreatment with 3-MA also increased the activation of NLRP3 inflammasomes and the protein levels of cleaved-caspase-1, IL-1β, and IL-18 relative to the SB216763 groups. Protein levels were normalized to β-actin. Quantification of the protein levels in (A) is shown in (B—H). All the data are represented as means ± SD. (MCAO vs. 3-MA and SB216763 vs. SB216763 + 3-MA,^$P > 0.05$; SB216763 vs. MCAO, #$P < 0.05$; SB216763 + 3-MA vs. MCAO, % P < 0.05; MCAO vs. 3-MA, @P < 0.05; SB216763 + 3-MA vs. SB216763, $$P < 0.05$). $n = 6$ per group.

3β also alleviated cerebral MCAO/R-induced injury by preventing NLRP3 inflammasome activation and increasing autophagic activity, as reflected by the increased ratio of LC3-II to LC3-I. It also decreased p62 levels. Finally, inhibiting autophagic activity by treating with 3-MA reversed the effect of GSK-3β inhibition on the NLRP3 inflammasome in cerebral I/R.

NOD-like receptors (NLRs) are a class of receptors or cytosolic sensors that respond to various pathogen-associated molecular patterns (PAMPs) linked to microbes and damage-associated molecular patterns (DAMPs) produced during injury. NLRP3 is one of the most studied

inflammasome receptors. It responds to changes in intracellular potassium levels and increased levels of reactive oxygen species and has a critical pathogenic role in neuroinflammatory disease [5,25]. Accumulating amounts of evidence have indicated a role for the NLRP3 inflammasome in neuronal cell death in ischemic stroke [27]. Our previous study showed that inhibiting the NLRP3 inflammasome exerted a neuroprotective effect against cerebral MCAO/R injury [3,25,27,28]. Above all, these findings indicate the importance of suppressing NLRP3 inflammasome activation for alleviating cerebral MCAO/R injury.

NLRP3 inflammasome activation requires two-step signaling: priming and assembly. Mitochondrial dysfunction acts upstream of NLRP3 activation by providing ROS and oxidized mitochondrial DNA to trigger NLRP3 inflammasome assembly [29]. Several studies have reported that autophagy and autophagy-related proteins play critical roles in regulating mitochondrial integrity, ROS generation, and subsequent NLRP3 activation [30]. Microtubule-associated protein-1 light chain-3 (LC3) and SQSTM1/p62 are two important components in the construction of autophagosomes [31]. During autophagy, the cytoplasmic form of LC3 (LC3-I; 18 kDa) is recruited to the autophagosome, where LC3-II (16 kDa) is produced by lipidation near the C-terminus and site-specific proteolysis [32]. p62 binds LC3 and recruits misfolded or aggregated proteins into autophagosomes for degradation [33]. The necessary process of autophagy is related to many diseases, including inflammatory, neurodegenerative, infectious, and neoplastic diseases [13]. Our previous study revealed that treatment with 3-MA could block resveratrol-mediated inhibition of the NLRP3 inflammasome [3]. The current study demonstrated that the level of autophagy increased ~24 h after MCAO and that the autophagy inhibitor 3-MA increased levels of the NLRP3 inflammasome and its downstream markers in cerebral MCAO/R injury.

GSK-3β is a serine/threonine kinase that plays an important role in many pathological and physiological processes in neurons. Inactivation of GSK-3β has multiple effects on promoting axonal growth and causes resistance to neurotoxin-induced cell death [36]. GSK-3 activity is facilitated by phosphorylation of tyrosine 216 in GSK-3β. The activation of this kinase in the midbrain and pons is likely to be involved in neuroinflammation induced by PQ [40]. Several studies have shown that the therapeutic effects of GSK-3β inhibitors are related to the inhibition of inflammation. The GSK-3β inhibitor SB216763 is a small molecule that competes with ATP and potently inhibits the activity of the α and β isozymes of GSK-3. It acts as neuroprotectant and prevents neuronal cell death induced by the PI3-kinase pathway [39]. We first addressed the effects of GSK-3βinhibitor SB216763 and siRNA on the total GSK-3βand the active form p-GSK-3β (Tyr216), and the results showed that SB216763 reduced the expression of p-GSK-3β (Tyr216) but the total GSK-3β.This indicates the activation not the expression of GSK-3βwas inhibited by SB216763. Huang et al. demonstrated that inhibiting GSK-3β induced secretion of the anti-inflammatory cytokine IL-10 [37]. In the current study, we aimed to investigate whether inhibiting GSK-3β attenuated cerebral MCAO/R injury by decreasing NLRP3 inflammasome activation. Previously, Wiekel reported that knockdown of GSK-3β increased autophagosome formation [19]. Ren et al. indicated that increased GSK-3β activity suppressed autophagy to promote the initiation and development of ALF by inhibiting the PPARα pathway [38]. Increasing evidence has shown that autophagy participates in I/R injury and plays a protective role against neuron damage [17,34]. Meng et al. found that the autophagy inhibitor 3-MA concurrently facilitated NLRP3 inflammasome activation and oxidation in fibroblasts [35]. To further explore the mechanism by which GSK-3β regulates the NLRP3 inflammasome, we hypothesized that inhibiting GSK-3β could suppress NLRP3 inflammasome activation by inducing autophagy. Our data revealed that inhibiting GSK-3β exerted protective effects against cerebral MCAO/R injury. In addition, treatment with GSK-3βinhibitor and siRNA suppressed NLRP3 inflammasome activation and cleaved-caspase-1, IL-1β, and IL-18 production compared with the MCAO group. Furthermore, GSK-3β inhibition increased the ratio of LC3-II to LC3-I, decreased the expression of p62, and increased the number of autophagosomes in cerebral I/R injury.

5. Conclusion

In summary, our data suggest that inhibition of GSK-3β protects against cerebral I/R injury by suppressing NLRP3 inflammasome activation and increasing autophagy. Although the underlying pathological mechanisms behind cerebral I/R injury have not been fully investigated, our study provides a possible novel direction for the treatment of stroke.

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