Introduction

Stroke is known to cause approximately 5.5 million deaths per year worldwide with more than 80 million stroke survivors suffering from cognitive and/or physical disabilities to varying degrees (Chamorro et al., 2021; Duncan et al., 2021). For managing stroke survivors, strategies focus on reducing disability by rapid reperfusion with intravenous thrombolytic agents or endovascular thrombectomy. With the expanded time window for intravenous thrombolysis from 4.5 hours up to 9 hours (Ma et al., 2012), approximately 15% of patients with stroke may be suitable for blood clot removal (Chia et al., 2021). Confronted with the challenges of stroke treatment, the underlying pathophysiology and mechanisms leading to ischemic insult have been illuminated comprehensively. For example, excitotoxicity, inflammation, glial activation, oxidative stress, and free radical or pro-inflammatory cytokine-mediated cytotoxicity have been reported to be involved in stroke pathology (Kuriakose and Xiao, 2020).

Recent studies have shown that β2-microglobulin (β2M), an approximately 12 kDa protein, affects multiple pathogenic conditions, such as kidney disease, some forms of cancer, and Alzheimer’s disease (Xie and Yi, 2003; Luton et al., 2013; Prizment et al., 2016). As a subunit of the major histocompatibility complex (MHC) class I molecule, β2M separates from MHC class I and regulates brain development, synaptic plasticity, and neurobehavior without independence from the canonical immune function (Huh et al., 2000; Boulanger and Shatz, 2004; Lee et al., 2014). Notably, soluble β2M in the cerebral spinal fluid (CSF) of patients with Alzheimer’s disease was higher than after stroke in rats (Boulanger and Shatz, 2004; Lee et al., 2014). Notably, soluble β2M in the cerebral spinal fluid, serum, and brain tissue were significantly increased in the acute period but gradually decreased during the recovery period. RNA interference was used to inhibit β2M expression in the acute period of cerebral stroke. Tissue staining with 2,3,5-triphenyltetrazolium chloride and evaluation of cognitive function using the Morris water maze test demonstrated that decreased β2M expression in the ischemic penumbra reduced infarct volume and alleviated cognitive deficits, respectively. Notably, glial cell, caspase-1 (p20), and Nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome activation as well as production of the inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α were also effectively inhibited by β2M silencing. These findings suggest that β2M participates in brain injury and cognitive impairment in a rat model of ischemic stroke through activation of neuroinflammation associated with the NLRP3 inflammasome.

Results

Increased β2M induced by ischemia-hypoxia is involved in brain damage and cognitive impairment by activating the NLRP3 inflammasome in a rat model of stroke

Discussion

Abstract

β2-Microglobulin (β2M), a component of the major histocompatibility complex class I molecule, is associated with aging-related cognitive impairment and Alzheimer’s disease. Although upregulation of β2M is considered to be highly related to ischemic stroke, the specific role and underlying mechanistic action of β2M are poorly understood. In this study, we established a rat model of focal cerebral ischemia by occlusion of the middle cerebral artery. We found that β2M levels in the cerebral spinal fluid, serum, and brain tissue were significantly increased in the acute period but gradually decreased during the recovery period. RNA interference was used to inhibit β2M expression in the acute period of cerebral stroke. Tissue staining with 2,3,5-triphenyltetrazolium chloride and evaluation of cognitive function using the Morris water maze test demonstrated that decreased β2M expression in the ischemic penumbra reduced infarct volume and alleviated cognitive deficits, respectively. Notably, glial cell, caspase-1 (p20), and Nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome activation as well as production of the inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α were also effectively inhibited by β2M silencing. These findings suggest that β2M participates in brain injury and cognitive impairment in a rat model of ischemic stroke through activation of neuroinflammation associated with the NLRP3 inflammasome.

Key Words: cognitive impairment; cognitive improvement; glial activation; infarct volume; ischemia; middle cerebral artery occlusion; neuroinflammation; NLRP3 inflammasome; stroke; β2 microglobulin

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After staining with eosin solution for 2 minutes, the sections were washed approximately 10 minutes. After washing, the samples were immersed in 100%, 95%, 80%, and 75% ethanol series, then stained with hematoxylin for 2 minutes, rinsed with double-distilled water, and then immersed in eosin for 1 minute. The escape latency, swimming speed, and the number of crossings over the previous platform area were captured on day 5 using a video system.

MCAO model was established by the intraluminal filament technique as previously described (Yao et al., 2009). The right external carotid artery was separated, a 19 mm filament (0.26 mm diameter; Guangzhou Jialing Biotechnology Limited; Guangzhou Province, China) was inserted into the right internal carotid artery via the external carotid artery stump, and the wound was sutured. For reperfusion, the filament was removed after 90 minutes of occlusion. The modified neurological severity scores (mNSS) test was used to evaluate neurological deficits 24 hours after MCAO. The mNSS test was performed using Morris water maze test at 4 weeks after surgery to assess cognitive function.

Rats were anesthetized with 2.5% isoflurane (Shandong Keyuan Pharmaceutical Co.; Jinan, China) in oxygen inhalation during the study.

The MCAO model was used to evaluate neurological and functional impairments. The rats were randomly divided into the sham, MCAO, lentivirus-recipient (LV-MCAO), and scramble control lentivirus-treated (SC) groups. The number of rats in each experiment is listed in Table 1. Rats were anesthetized with 2.5% isoflurane (Shandong Keyuan Pharmaceutical Co.; Jinan, China) in oxygen inhalation during the study. The filament was not inserted into the artery of sham rats. A flow chart of this study design is shown in Figure 1.

**Lentiviral production and stereotactic surgery**

The shRNA target sequence 5’-ACC GAT GTA TAT GTG TCG A-3’ for rat β2M and the control sequence 5’-TTC TCC GAA CGT GTC ACG T-3’ were amplified and inserted into GV118 vectors to form β2M recombinant plasmids and control sequence plasmids. The recombinant and lentiviral helper plasmids were used to co-transfect HEK293 T cells for 16 hours. The cells were harvested, filtered, and concentrated. The Western blotting analysis of the LV-β2M-RNAi was 3 × 10^7 infectious units/ml. Lentiviral production was performed by Shang Hai Genechem Co. (Shanghai, China). For stereotactic injection of lentivirus into the brain, rats were anesthetized, positioned, and a small hole was created in the skull. A total of 1, 1 μL lentivirus was injected into the brain site relative to the bregma (anterior-posterior, +2.5 mm; medial-lateral, ±1.7 mm; dorsal-ventral, −2.5 mm) using a Hamilton syringe needle (33 gauge; Hamilton; Bonaduz, Switzerland). The needle was removed after 10 minutes.

**Morris water maze test**

As previously described (Fan et al., 2017), the rats were subjected to the Morris water maze test at 4 weeks after surgery to assess cognitive function. Briefly, each rat was trained to find the submerged escape platform each day for 4 days. On day 5, the rats swam in the water tank without the platform for 1 minute. The escape latency, swimming speed, and the number of crossings over the previous platform area were captured on day 5 using a video system (RWD Life Science; Shenzhen, China). The MCAO model was used to evaluate neurological and functional impairments.

**Hematoyxlin-eosin staining**

At 7 days after MCAO, rats were perfused, and brain samples were collected, paraffin-embedded, and sectioned using a microtome (Leica Microsystems; Wetzlar, Germany). To observe pathological changes, 5-μm coronal sections were stained with hematoxylin-eosin in accordance with the manufacturer’s instructions (Beyotime Biotechnology Inc.; Shanghai, China). Briefly, the samples were dewaxed with xylene, washed using a 100%, 95%, 80%, and 75% ethanol series, then stained with hematoxylin for approximately 10 minutes. After washing, the sections were immersed in 100% ethanol and then washed with tap water. After staining with eosin solution for 2 minutes, the sections were washed and dehydrated with graded ethanol and sealed with neutral resin.

**Immunofluorescent staining**

As previously reported (Fan et al., 2017), rats were transcardially perfused with 0.9% NaCl solution at 3 hours, and 1, 3, 7, or 14 days after MCAO. After post-fixing in 4% paraformaldehyde for several hours, the brains were placed in 15% sucrose. 3% (w/v) Phosphate buffer overnight and then in 30% sucrose until water was replaced completely. Serial cryosections (20 μm) were prepared on poly-lysine-coated slides for immunofluorescent staining. For western blots, the ischemic penumbra of each brain was separated, weighed, and then homogenized in cold lysis buffer supplemented with protease and phosphatase inhibitors (1:3 [w/v]). After sonicating, the homogenates were centrifuged, and the supernatants were aliquoted and maintained in an ultra-cold storage freezer (Thermo Fisher Scientific; Waltham, MA, USA). After blocking in 3% normal donkey serum (Abbkine Scientific Co., Wuhan, China) for 1 hour at room temperature, the sections were incubated with one of the following primary antibodies overnight at 4°C: anti-β2M (mouse; 1:100; Santa Cruz Biotechnology, Dallas, TX, USA, Cat# sc-22421, RRID: AB_2662750), anti-gliarial fibrillary acidic protein (GFAP; maker for astrocytes; rabbit; 1:500; Proteintech, Cat# 16825-1-AP, RRID: AB_2106646; Chicago, IL, USA), anti-NeuN (marker for neurons; rabbit; Cell Signal Technology, Boston, MA, USA, Cat# 12943S, RRID: AB_2630935), and anti-ionized calcium-binding adaptor molecule 1 (Iba-1; marker for microglia; rabbit; Fujifilm Wako Shibayagi, Osaka, Japan, Cat# 016-20010, RRID: AB_839506). The sections were washed several times and incubated with the secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, USA). The sections were washed and incubated with 4',6-diamidino-2-phenylindole (Beyotime Biotechnology Inc.) to label the nuclei. Images were captured using a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany). The numbers of positive cells were quantified using Image-Pro Plus software.

**Western blotting analysis**

For protein analysis, brain tissue protein was extracted using tissue lysis buffer supplemented with protease inhibitors. After the protein concentration was determined, the lysates were separated on 10% or 15% polyacrylamide gels by electrophoresis. After electrophoresis, the blotting membrane (0.45 μm nitrocellulose) was trimmed to fit the size of the gel. For electrophoretic transfer, the blotting paper was soaked in a cold tris-buffer, and the current strength was set at 350 mA and time for western blotting was approximately 70–120 minutes according to the protein molecular weight. After transferring proteins to the membranes, the membranes were blocked with one of the following blocking agents at room temperature for 1 hour: 5% nonfat milk or 5% skim milk in tris-buffer. The sections were washed and incubated with 4',6-diamidino-2-phenylindole (Beyotime Biotechnology Inc.) to label the nuclei. Images were captured using the Odyssey infrared imager (LI-COR, Lincoln, NE, USA) and quantified using the optical density values.

**Enzyme-linked immunosorbent assay**

At 3 hours, and 1, 3, 7, and 14 days after MCAO, venous blood was collected from the rats into serum tubes from the angular vein using a blood collection needle (Roche; Basel, Switzerland). The blood was coagulated for 30 minutes at 37°C. Serum was collected and stored at −70°C. CSF was collected according to a minimally-invasive method (Simat et al., 2018). Briefly, the rats were anesthetized and properly positioned on a stereotactic apparatus (RWD Life Science). A needle (25 gauge), which was connected to a collection tube, was vertically and slowly inserted into the cisterna magna. To avoid blood contamination, the tube was clipped when the collection was almost finished, and the needle was withdrawn slowly. β2M concentrations in sera and CSF were measured by an enzyme-linked immunosorbent assay kit (Dianova Biotech Co., Ltd., Wuhan, China) following the manufacturer’s protocol.

**2,3,5-Triphenyl tetrazolium chloride staining**

Rats were perfused with 2% 3,5-triphenyl tetrazolium chloride in accordance with the manufacturer’s protocol. At 3 days after MCAO, the brains were collected and serial slices (2 mm thick) were prepared. The slices were stained in 2% 3,5-triphenyl tetrazolium chloride solution (Sigma-Aldrich; St. Louis, MO, USA) for 30 minutes and washed completely with double-distilled water. The infarct areas were photographed and measured using ImageJ software (National Institutes of Health; Bethesda, MD, USA) (Schneider et al., 2012).
Statistical analysis
According to pilot studies and a previous research study (Yao et al., 2009), we chose a proper hypothesis and simplified the studies by using numeric results for testing two proportions and the Z-test with unpoled variance for sample size calculation. Statistical analyses were performed with GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) by observers blinded to the experimental design. Two-way analysis of variance was used when the subjects are subjected to repeated measures, and one-way analysis of variance followed by Tukey’s honestly significant difference post hoc test was used to test for more independent groups. Data are presented as means ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

Results
The β2M levels are increased in the serum, CSF, and brain tissue in the rat stroke model
We performed enzyme-linked immunosorbent assays, immunofluorescent staining, and western blots to determine whether ischemic-hypoxic brain injury resulted in changes in β2M levels in sera, CSF, and brain tissue at 3 hours and 1, 3, 7, and 14 days post-MCAO. CSF (P < 0.0001 at 3 hours, P = 0.024 at 1 day) and serum β2M (P < 0.0001 at 3 hours, P = 0.016 at 1 day, P = 0.010 at 3 days, P = 0.0071 at 7 days) levels were significantly elevated in the model rats, peaked at 3 hours after MCAO, and gradually returned to normal levels at 3 and 14 days, respectively (Figure 2A and B). In addition, the protein expression of β2M (P = 0.02 at 3 hours, P < 0.0001 at 1 day, P = 0.0179 at 3 days; Figure 2C and D) and β2M-positive cell numbers (P < 0.0001 at 3 hours, P < 0.0001 at 1 day, P < 0.0001 at 3 days, P = 0.0011 at 7 days, P = 0.0035 at 14 days; Figure 2E and F) were also markedly increased in the ischemic penumbra. As shown in Figure 2G, β2M was mainly expressed in NeuN-positive neurons. Our results suggest that ischemic stroke leads to an enhancement of β2M expression in rats.

B2M knockdown decreases the infarct volume and improved cognitive deficits in the rat stroke model
Cerebral infarct volume of the MCAO rats was decreased by B2M knockdown as observed with triphenyl-2,3,5-tetrazolium chloride staining (P = 0.0033, Figure 4). During the 4-day training portion of the Morris water maze test, MCAO rats showed longer escape latency (P < 0.0001 on day 3 and P = 0.0001 on day 4), and B2M knockdown significantly decreased the latency period compared with that in the SC group on day 4 (P = 0.0399, Figure 5A and B). On day 5, the swimming time for MCAO rats in the target quadrant was less than that of sham rats (P = 0.0005), whereas B2M knockdown increased the swimming time compared with that for the SC lentivirus-treated rats (P = 0.0445, Figure 5C). Moreover, B2M knockdown significantly increased the number of crossings over the original platform location (P = 0.0462; Figure 5D) without affecting the swimming speed (Figure 5E). These results indicated that inhibition of B2M expression improved the cognitive function of MCAO rats.

Lentiviral-mediated RNA interference silences B2M expression effectively in the rat stroke model
To explore the role of B2M in neurological function and neuroinflammation of MCAO rats, RNA interference was used to silence the B2M gene. Both the number of B2M-positive cells (P = 0.0011; Figure 3A and B) and B2M protein levels (P = 0.0002; Figure 3C) were decreased in the ischemic penumbra of the MCAO rats after B2M shRNA treatment compared with those in the SC shRNA-treated rats.
β2M knockdown inhibits glial activation after cerebral ischemia.

Western blot analysis showed that protein levels of NLRP3 and ASC (an NLRP3 inflammasome constituent) were notably increased (P < 0.0001 for NLRP3; P < 0.0001 for ASC) in ischemic cerebral cortexes of the MCAO model rats compared with those of sham rats (Figure 7A and B). In addition, caspase-1 (p20) (P = 0.0004), IL-1β (P = 0.0003), IL-6 (P < 0.0001), and TNFα (P = 0.0002) were significantly upregulated in the MCAO group compared with the sham group. SC lentivirus treatment did not reverse the outcomes of the model rats (Figure 7A–C).

Inhibition of β2M alleviates NLRP3 inflammasome activation and neuroinflammation in the rat stroke model

Studies have indicated that neuroinflammation plays a significant role in cerebral ischemia and may cause neural damage by promoting cell death or induce beneficial stimulation of remedial action. Here, we investigated whether β2M is involved in the pathophysiology of ischemic stroke by activating the NLRP3 inflammasome and pro-inflammatory cytokine production. Our results demonstrated that ischemia-hypoxia caused a significant increase in β2M and NLRP3 inflammasome activation; β2M knockdown inhibited NLRP3 inflammasome activation and inflammatory reactions. Furthermore, silencing β2M resulted in smaller infarct size and improved cognitive function. Our study established the detrimental effects of β2M in ischemic stroke.

Accumulating evidence suggests that β2M, a light chain of approximately 100 amino acids that is co-expressed with MHC class I molecules (Zijlstra et al., 1990), is closely related to neurological disorders. Serum β2M was significantly increased in schizophrenia patients compared with healthy controls, and the level of β2M was positively correlated with the total psychopathology score (Chuttong et al., 2009). In addition, soluble β2M in the CSF was elevated in patients with dementia (McArthur et al., 1992), Parkinson’s disease (Carrette et al., 2003), and Alzheimer’s disease (Carrette et al., 2003; Zhang et al., 2008). Moreover, high levels of β2M resulted in cognitive function impairment and neurogenesis inhibition (Smith et al., 2015) and directly induced depressive-like behaviors in rats (Zhang et al., 2018a). In recent years, clinical studies have demonstrated that increased serum β2M is highly associated with ischemic stroke (Rist et al., 2017; Hu et al., 2019; Qun et al., 2019). Using a cerebral ischemia model, we revealed a dramatically time-dependent increase in the levels of β2M in serum, CSF, and brain tissue of rats. β2M was secreted by most nucleated cells, especially inflammatory cells. Ischemic stroke is accompanied by destruction of the blood-brain barrier. We infer that β2M is mainly secreted by inflammatory cells, which leads to abnormally high levels of serum and CSF β2M in the acute stage of ischemia. However, in the brain parenchyma, β2M transcription, translation, and secretion were delayed in neuronal cells. Thus, the level of β2M expression peaked on day 1 in the brain but peaked at 3 hours in the CSF and serum after MCAO. These
observations suggest that β2M abnormalities may be potentially associated with the pathogenesis of ischemic stroke.

Previous studies provided evidence that the β2M level is negatively associated with cognitive performance (Smith et al., 2015; Gao et al., 2018; Chen et al., 2019). To address the role of β2M in cognitive impairment induced by ischemia-hypoxia, we reduced the level of β2M using shRNA cloned into a lentivirous vector. The Morris water maze test confirmed the detrimental effects of β2M on learning and memory function. Moreover, we observed that β2M knockdown reduced the infarct volume of MCAO rats, suggesting that increased β2M after MCAO has a detrimental effect on cognition and brain function. However, the mechanisms of β2M involvement in cognitive impairment remain unclear.

More recent studies have demonstrated that the NLRP3 inflammasome is a critical mediator of inflammatory responses after stroke. Inhibiting NLRP3 considerably prevented a cascade of inflammatory reactions and attenuated neuronal damage after ischemia/reperfusion injury in animal models (Deroide et al., 2013; Yang et al., 2014; Han et al., 2020), suggesting that the NLRP3 inflammasome is a potential therapeutic intervention target for ischemic stroke (Feng et al., 2020). It has been reported that β2M is a marker of low grade inflammation (Raikou and Kyriaki, 2015; Wu et al., 2017). Therefore, we determined whether β2M was associated with a corresponding change in inflammation within the brain. As expected, β2M silencing reversed NLRP3 inflammasome activation, pro-inflammatory cytokines production, and glial activation, suggesting that β2M is involved in the neuroinflammatory reaction after stroke.

Pro-inflammatory cytokines promote the progression of harmful neuroinflammation after cerebral ischemia. As one of the first cytokines to be produced, TNFα increases in the cerebral parenchyma following MCAO (Liu et al., 1994) and triggers secondary inflammatory reactions (Murakami et al., 2003). Following cerebral ischemia, IL-1β binds to its receptor and activates the nuclear factor-kB pathway (Liao et al., 2015). IL-1β knockout significantly alleviated cerebral damage of mice after MCAO (Ohtaki et al., 2003); however, administration of IL-1β exacerbated cerebral ischemic injury (Yamasaki et al., 1995). Following cerebral ischemia, IL-6 increased markedly in serum within several hours and persisted for up to 90 days (Herrmann et al., 2003). Because neuroinflammation definitively and adversely affects cognitive function (Kuile et al., 2016), we propose that β2M inhibition protects the brain, to a certain extent, against ischemic damage by reducing β2M production.

In conclusion, increased β2M plays a role in neuroinflammation and cognitive impairment after ischemic stroke. Inhibition of β2M is beneficial for improving repair of cerebral ischemic injury and cognitive impairment, and the mechanism is related to the reduction of NLRP3 inflammasome activation and inflammatory cytokine production. There were some deficiencies in this study. For example, we did not add recombinant β2M back to the knockdown rats to examine whether the phenotype was rescued. We also did not observe whether the outcomes were worse if β2M was overexpressed or exogenously administrated to the rats. Clarification of these issues will require further investigations.

Author contributions: Study design: RQY; experiment implementation: YL, FFH, RQY; data analysis: FC, JL, FQL, SSW, YYZ, YYL, FFH; data visualization: JL, FFH; manuscript draft: FC, JL, RQY. All authors approved the final version of this paper.

Conflicts of interest: The authors declare that they have no conflicts of interest.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Additional file: Additional Table 1: The number of animals or samples in every experiment.

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### Additional Table 1 The number of animals or samples in every experiment

| Experimental methods       | Number of animals/samples each group (n) |
|----------------------------|----------------------------------------|
| Morris water maze         | 12                                     |
| HE staining                | 4                                      |
| TTC staining               | 6-8                                    |
| Immunofluorescent staining | 4                                      |
| Western blot               | 4                                      |
| ELISA                      | 9                                      |

ELISA: Enzyme-linked immunosorbent assay; HE: hematoxylin-eosin; TTC: triphenyl-2,3,5-tetrazoliumchloride.