CXCL-12 Attenuates Neuroinflammation via the CXCR4/PI3K/Akt Signaling Pathway in a Rat Model of SAH

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Abstract

Background

Subarachnoid hemorrhage (SAH) is a cerebrovascular disease associated with high morbidity and mortality. CXCR4 provides a neuroprotective effect, which can alleviate brain injury and inflammation induced by stroke. The purpose of this study was to evaluate the anti-inflammatory effects and mechanisms of CXCR4 after SAH. Methods: SAH was induced via endovascular perforation. 185 male Sprague-Dawley rats were used. Recombinant human cysteine-X-cysteine chemokine ligand 12 (rh-CXCL-12) was administered intranasally at 1 h after SAH induction. To investigate the underlying mechanism, the inhibitors of CXCR4 and P13K, AMD3100 and LY294002, respectively, were administered intraperitoneally at 1 h before SAH. The short- and long-term neurobehavior were assessed, followed by performing western blot and immunofluorescence staining.

Results

Western blotting suggested that the expressions of endogenous CXCL-12 and CXCR4 were increased, and peaked at 24 h following SAH. Immunofluorescence staining showed that CXCR4 was expressed on microglia. Rh-CXCL-12 treatment reduced the number of M1 macrophages and improved the short- and long-term neurological deficits after SAH. Meanwhile, rh-CXCL-12 treatment increased the levels of CXCL-12, CXCR4, PI3K, and p-Akt, and reduced the levels of IL-1 β , IL-6, and TNF- α . Moreover, the administration of AMD3100 and LY294002 abolished the post-SAH neurobehavioral and neuroinflammatory improvements of CXCL-12 and its regulation of PI3K and p-Akt protein levels.

Conclusions

The CXCR4/PI3K/Akt signaling pathway may be involved in CXCL-12-mediated reduction of post-SAH neuroinflammation. Early administration of CXCL-12 may be a preventive and therapeutic strategy against delayed brain injury after SAH.

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a devastating and life-threatening disease associated with high mortality and disability [1]. The annual worldwide incidence of SAH is approximately 9.1 per 100,000 people [2], and SAH resulting from intracranial aneurysm rupture accounts for 5% of all strokes [3]. Early brain injury (EBI) appears in the first 3 days following SAH, and is the major cause of poor prognosis regarding the high mortality and delayed neurological deficits [3].

EBI begins immediately after a ruptured intracranial aneurysm, and has been proven to be the primary cause of poor outcomes after SAH. During the EBI period, ruptured intracranial aneurysm resulted in many physiological derangements, including elevated intracranial pressure, decreased cerebral blood flow, and global cerebral ischemia [4]; all of which initiate a variety of pathophysiological events, such as

oxidative stress, neuroinflammation, blood–brain barrier dysfunction, and apoptosis [5,6]. Neuroinflammation induced by SAH has been considered a main devastating pathophysiological process in EBI after SAH [7].

Microglia, the resident immunecellsofthecentralnervoussystem(CNS), play an important role in regulating neuroinflammation [8]. In a healthy brain, microglia are usually in a resting status (M0). However, in the disease state, microglia adopt two different activation phenotypes: the pro-inflammatory and neurotoxic "classical" activation (M1) phenotype and the "alternative" activation (M2) phenotype with anti-inflammatory and neuroprotective effects (M2) [9]. In the early stages, microglia are expressed as the M1 phenotype, which secretes proinflammatory cytokines, such as IL-1, IL-6, and TNF- α to damage brain tissue in EBI after SAH. In the late stage of SAH, the M2 phenotype plays a major role in reducing inflammation and tissue injury [10,11]

Cysteine-X-cysteine chemokine ligand 12 (CXCL-12), is an inflammatory chemokine derived from bone marrow mesenchymal stem cells, and belongs to the CXC chemokine family. CXC chemokine receptor type 4 (CXCR4) is one of seven transmembrane G-protein-coupled receptors that mediates transmembrane signaling of CXCL-12. CXCL-12 and CXCR4 are abundant and widely expressed in CNS, playing an important role in neurogenesis and contributing to the neuronal development [12]. CXCL-12 is known to be expressed in neurons, glial cells, endothelial cells, and meningeal cells [13]. Previous studies have shown that CXCL-12 is upregulated in the penumbra after stroke, reduces neurological deficits, and promotes neuroprotection and angiogenesis [14–16]. CXCR4 is reportedly expressed in neurons, astrocytes, microglia, and ependymal cells [17]. Recent studies have demonstrated that CXCR4 is involved in the inflammatory response and exerts neuroprotective effects after ischemic stroke [18,19]. However, there is currently no research exploring the role of CXCR4 and the underlying mechanism after SAH. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) is the downstream of CXCL-12/CXCR4 axis [20]. PI3K/Akt receives a signal from the G-protein-coupled receptor, CXCR4. P85 and P110, the two subunits of PI3K, were activated by conformational changes in space, which led to phosphorylation of Akt and activation of the pathway [21]. Recently studies have shown that the PI3K/Akt signaling pathway plays an important role in mediating cell survival, differentiation, proliferation, apoptosis, and metastasis [22]. Further evidence suggests that activation of the PI3K/Akt signaling pathway provides neuroprotective effects, which can reduce the cerebral infarct area and neuronal damage, and attenuate apoptosis in the ischemic penumbra [23]. PI3K/Akt also improves the neurobehavioral defects of SAH by reducing neuroinflammation and BBB destruction [24].

In the present study, we hypothesized that rh-CXCL-12 would attenuate neuroinflammation by inhibiting microglial activation, reducing pro-inflammatory cytokine secretion, and improving neurological impairments. Moreover, these beneficial effects were, at least in part, via up-regulation of CXCR4/PI3K/Akt signaling pathway after SAH in rats (Fig. 1).

Materials And Methods

Animals

Adult male Sprague–Dawley rats (weighting 280–330g, n=185) obtained from Guizhou Laboratory Animal Engineering Technology Center (China) were used in this project. All animals were kept in a room with controlled humidity ($60 \pm 5\%$), constant temperature ($25 \pm 1^\circ\text{C}$), and remained in a 12 h light and dark cycle and with ad libitum access to food and water.

SAH model

The SAH model was performed in rats using a modified endovascular perforation model as previously described [25]. Induction of anesthesia in rats was achieved using 4% isoflurane, and was maintained using 2.5% isoflurane. After intubation, the mice were placed in the supine position and connected to the rodent ventilator to breathe medical air (70%) and oxygen (30%). The heart rate, respiration, skin color, and pedal reflex assessment were assessed every five minutes during the operation to confirm anesthesia status and prevent distress. After exposing the carotid artery and its bifurcation, a 4-0 sharp single nylon thread suture was inserted from the external carotid artery into the left internal carotid artery to the anterior and middle cerebral artery bifurcation. The nylon suture was withdrawn immediately, and isoflurane was reduced to 1.5%. After the operation, the endotracheal tube was removed and the animals were placed in the heating chamber (37.5°C) to recover. Animals in the sham group underwent the same procedure, but without arterial wall puncture.

SAH Grading

The degree of SAH was assessed according to the SAH grading scale system at 24 h after SAH as previously described [26]. The basal cistern was divided into six segments that were graded from 0 to 3 according to the amount of subarachnoid blood. The total score was calculated by adding all area scores (maximum SAH grade = 18). Rats with a score of 8 or less were excluded from the current study.

Experimental design

Four separate experiments were performed as shown in Fig. 2.

Experiment 1

To determine the time course of endogenous CXCL-12 and CXCR4 protein level expression in the sham group and each group after SAH. The rats were randomly divided into six groups (n=6/group): Sham, SAH-6 h, SAH-12 h, SAH-24 h, SAH-48 h, and SAH-72 h. Western blot analysis was performed to assess the protein levels of CXCL-12 and CXCR4 in the ipsilateral (left) hemisphere cerebral cortex. Additionally, the cellular localization of CXCR4 with calcium-binding adaptor molecule 1 (Iba-1) was evaluated using double immunofluorescence staining in the Sham and SAH-24 h group (n=2/group).

Experiment 2

To evaluate the neuroprotective effects of CXCL-12 on short-term neurological outcomes after SAH, rats were randomly assigned to five groups (n=6/group): Sham, SAH+vehicle (sterile distilled water), SAH+CXCL-12 (5 µg/kg), SAH+CXCL-12 (15 µg/kg), and SAH+CXCL-12 (45 µg/kg). CXCL-12 was administered intranasally (i.n.) at 1 h after SAH. The SAH grading score, neurobehavioral test (including modified Garcia test and beam balance test), and brain water content were assessed at 24 h after SAH in all groups. The best dose of CXCL-12 was selected based on the short-term neurological outcomes and brain water content results, which was also used for the following long-term outcome and mechanism experiments.

To explore the effects of CXCL-12 on microglia/macrophage activation and neutrophil infiltration at 24 h after SAH, rats were randomly assigned to three groups (n=4/group): Sham, SAH+vehicle (sterile distilled water), and SAH+CXCL-12 (optimal dose). Immunofluorescence staining of Iba-1 with CD68 and myeloperoxidase (MPO)-positive neutrophils was performed in the peri-hemorrhagic area at 24 h after SAH. Quantitative analysis of CD68-positive Iba-1 and relative fluorescence density of MPO were assessed. Brain samples of these three groups were shared with experiment 4.

Experiment 3

To evaluate the effects of CXCL-12 on long-term neurobehavioral outcomes after SAH, rats were randomly assigned to three groups (n=10/group): Sham, SAH+vehicle (sterile distilled water), and SAH+CXCL-12 (optimal dose). The Rotarod test was performed on days 7, 14, and 21 after SAH. Morris water maze was performed on days 23–27 after SAH.

Experiment 4

To explore the underlying mechanism of the CXCR4/PI3K/Akt signaling pathway-mediated anti-inflammatory effects after SAH, the selective CXCR4 inhibitor, AMD3100, and PI3K inhibitor, LY294002, were administered intraperitoneally (i.p.) at 1 h before SAH. Rats were randomly assigned to seven groups (n=10/group): Sham, SAH+Vehicle (sterile distilled water, i.n.), SAH+CXCL-12, SAH+CXCL-12+AMD3100, SAH+CXCL-12+PBS (Vehicle of AMD3100), SAH+CXCL-12+LY294002, and SAH+CXCL-12+DMSO (Vehicle of LY294002, i.p.). The ipsilateral (left) hemisphere of each group was collected for western blot analysis (n=6/group) and immunofluorescence staining (n=4/group) after neurological performances and SAH grades were evaluated at 24 h after SAH.

Drug administration

CXCL-12 or vehicle was given via intranasal administration at 1 h after SAH as previously described [27]. Animals were placed in the supine position and were administered 1.5% isoflurane anesthesia. A total volume of 20 µL of vehicle (sterile distilled water) or CXCL-12 (MedChem Express, NJ, USA) at three different doses (5 µg/kg, 15 µg/kg, and 45 µg/kg), with 5 µL administered every 5 minutes, alternating between the right and left nares. AMD3100 was diluted in PBS, LY294002 was diluted in 10% dimethyl sulfide (DMSO), and both were administered intraperitoneally (i.p.) at 1 h before SAH.

Assessment short-term neurological performance

The short-term neurobehavioral outcomes were assessed blindly using the 18 point modified Garcia scoring system and the 4 point beam balance test at 24h after SAH as previously described [28]. Higher scores indicated better neurological function.

Assessment long-term neurological performance

Long-term neurobehavioral effects were assessed using the rotarod test to evaluate sensorimotor coordination and balance on days 7, 14, and 21 after SAH, and the Morris water maze was used to evaluate spatial learning capacity and memory ability on day 23–27 after SAH as previously described [29]. For the rotarod test, the animals were placed on the rotarod at the starting rate of 5 revolutions per minute (RPM) or 10 RPM, followed by gradual acceleration of 2 RPM every 5 seconds. The duration that the rats were able to stay on the accelerating rotating cylinder was recorded and used for statistical analysis. In regard to the Morris water maze test, animals were taken to the platform on the first day of cueing test. For the spatial learning test in the following days, the animals were placed in a set of semi-random starting positions, and were tasked to find the submerged platform within the 60-second time limit. The probe test was performed with the actual platform removed on day 27 after SAH. Swimming distance and trace, escape latency, and probe quadrant duration were recorded by the Computer Tracking System (San Diego Instruments Inc., CA, USA).

Brain water content

Brain edema was assessed by measuring brain water content using the wet-dry method as previously described [6]. The rats were euthanized at 24 h after SAH, and the brains were quickly removed and separated into four parts (right hemisphere, left hemisphere, cerebellum, and brain stem). Afterwards, each part of the brain was weighed immediately to obtain the wet weight, and then placed into an oven for 72 h at 100°C. The dried brain was weighed again. The percentage of brain water content was calculated as follows: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$.

Immunofluorescence staining

The rats were deeply anesthetized (5% isoflurane), and were euthanized via trans-cardiac perfusion with 100-150 mL of pre-cooled PBS (4 °C) and 100 mL of 10% formalin. Whole brains were rapidly collected and fixed in 10% formalin (4 °C, 24 h), followed by dehydration with 30% sucrose (4 °C, 72 h). Brain samples were embedded in OCT (Scigen Scientific Gardena, CA, USA), and then frozen at – 80 °C. The brains were sliced into 10 µm thick coronal brain sections using a cryostat (CM3050S, Leica Microsystems, Bannockburn, Germany), and then mounted onto normal Poly-L-Lysine coated slides. The slices were washed with 0.01 M of PBS three times for 5-10 min, and then incubated in 0.3% Triton X-100 in 0.01 M of PBS for 10 min at room temperature. After being blocked with 5% donkey serum in 0.01 M of PBS for 2 h at room temperature, the sections were incubated overnight at 4 °C with the following primary antibodies: anti-Iba-1 (1:200, Abcam), anti-CXCR4 (1:200, Abcam), anti-CD68 (1:200, Santa Cruz

Biotechnology), rabbit anti-IL-1 β (1:200, Abcam), and mouse anti-MPO (1:200, Santa Cruz Biotechnology). Next, the slices were incubated with fluorescence-conjugated secondary antibodies (1:500, Jackson ImmunoResearch) for 1 h at room temperature. The slides were visualized and photographed using a fluorescence microscope (DMI8, Leica Microsystems). The number of CD68-positive microglia cells were identified and counted in three different fields from the left basal cortex of five random coronal sections of each rat. The positive cells were quantified under microscopic field of 200x magnification, and data were expressed as cells/field. To assess neuroinflammation levels, six randomly selected tubules were examined to count IL-1 β - and MPO-positive cells under the microscopic field at 400x and 200x magnification. The fluorescence intensity was quantified by ImageJ software (ImageJ 1.5, NIH, USA).

Western blot analysis

At 24 h after SAH, rats were deeply anesthetized (5% isoflurane) and transcardially perfused with chilled PBS, followed by decapitation. The brain sections were separated into ipsilateral and contralateral hemispheres. The ipsilateral hemisphere brain tissues were snap frozen in liquid nitrogen and stored in -80 °C freezer for storage until used. Brain samples were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) with protease inhibitor for 15 min and then centrifuged at 14,000 g (4 °C, 30 min). The supernatant was collected, and protein concentration was measured by detergent compatible assay (DC Protein Assay, Bio-Rad Laboratories). Equal amounts of protein were loaded onto the 10% SDS-PAGE gel for electrophoresis and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat blocking grade milk (Bio-Rad) for 2 h (37 °C), and incubated overnight at 4 °C with the following primary antibodies: anti-CXCR4 (1:1000, Abcam), anti-CXCL-12 (1:1000, Abcam), anti-p-Akt (1:1000, Cell Signaling Technology), anti-Akt (1:1000, Cell Signaling Technology), anti-PI3K (1:1000, Thermo Fisher Scientific), anti-IL-1 β (1:1000, Abcam), anti-IL-6 (1:1000, Abcam), anti-TNF- α (1:1000, Abcam), and anti- β -actin (1:5000, Santa Cruz Biotechnology). The membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology) for 1h at 37°C. The bands were then visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Pittsburgh, PA) and quantified with the ImageJ software (ImageJ 1.5, NIH, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (Graph Pad Software, San Diego, CA, USA). All data were presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's post-hoc test was used for comparison among multiple groups. Two-way ANOVA was used to analyze the long-term neurobehavioral results. $P < 0.05$ was considered statistically significant.

Results

Mortality and SAH grading score

Of the 185 rats used, 151 rats underwent SAH induction. Of which, 21 (14.89%) rats died within 24 h after SAH, and 10 rats were excluded from this project due to mild SAH. There was no mortality in the Sham

group (Fig. s1). Subarachnoid blood clots were distributed around the circle of Willis and ventral brain stem after SAH induction, with a significant difference in sham group (Fig. 4 a). The average SAH grading scores among all SAH groups showed no significant differences (Fig. 4 b).

Expression levels of endogenous CXCL-12 and CXCR4, and colocalization of CXCR4 with microglia after SAH

As shown in Fig. 3, the results of western blotting showed that the endogenous protein expression levels of CXCL-12 and CXCR4 increased in a time-dependent manner, and peaked at 24 h after SAH when compared to the sham group ($P < 0.05$, Fig.3 a-c). Co-immunofluorescence staining of Iba-1 with CXCL-12 showed that CXCL-12 was expressed on the microglia within cortices in the sham group and in the perihemorrhagic area at 24 h after SAH (Fig.3 d).

Intranasal administration of exogenous rh-CXCL-12 improved short-term neurobehavioral dysfunctions and attenuated brain edema at 24 h after SAH

The neurobehavioral outcomes of modified Garcia and beam balance were significantly reduced at 24 h after SAH in the SAH+vehicle and SAH+rh-CXCL-12 (5 µg/kg) groups. However, administration of rh-CXCL-12 (15 µg/kg) and rh-CXCL-12 (45 µg/kg) significantly improved the neurological scores at 24 h after SAH ($P < 0.05$, Fig.4 c, d). The brain water content in the left and right hemisphere was significantly increased in the SAH+vehicle and SAH+rh-CXCL-12 (5 µg/kg) group, which was significantly reduced by the administration of rh-CXCL-12 at doses of 15µg/kg and 45 µg/kg ($P < 0.05$, Fig. 4 e). Brain water contents in the cerebellum and brain stem were not significantly different between the sham and SAH groups. Based on these results, the optimal dose of rh-CXCL-12 was 15µg/kg, which was used for the following long-term and mechanistic studies.

Rh-CXCL-12 reduced microglial activation and neutrophilic infiltration at 24 h after SAH

Iba-1 is constitutively expressed in both resting and active microglia, while CD68 is only labeled the activated microglia (*M1 phenotypemicroglia*). Double-immunofluorescence staining of Iba-1 with CD68 were performed to evaluate microglial activation in the ipsilateral basal cortex at 24 h after SAH. Compared to the sham group, rats in the SAH+vehicle group showed an increase in Iba-1 positive cells with activated microglial morphology of rod shape or larger body with short/thick processes. Rats treated with rh-CXCL-12 had fewer CD68-positive activated microglia at 24 h after SAH (Fig. 5 a). Quantitative analysis showed that rh-CXCL-12 administration significantly reduced the number of CD68-positive activated microglia (Fig. 5c). Moreover, the number of MPO-positive cells were significantly increased in the SAH+vehicle group (Fig. 5 a). Lastly, the rh-CXCL-12 treatment significantly reduced the number of MPO-positive cells (Fig. 5 d).

Rh-CXCL-12 improved long-term neurological deficits after SAH

The rotarod test results revealed that the rats had significantly shorter falling latency at both 5 and 10 rpm in the SAH+vehicle group when compared with the sham group in the 1st, 2nd, and 3rd weeks after SAH. However, administration of rh-CXCL-12 improved the rotarod performance of SAH rats compared to the SAH+vehicle group ($P < 0.05$; Fig. 6 a, b). The results of the water maze test showed that the escape latency and travel distance for the rats to find the platform were remarkably increased in the SAH+vehicle group when compared to the sham group. However, a significantly shorter distance and time to find the platform were observed in the SAH+rh-CXCL-12 group ($P < 0.05$; Fig. 6 d, e). In the probe quadrant trial, the rats in the SAH+vehicle group remained in the target quadrant for a shorter period when compared to the sham group. However, rh-CXCL-12 significantly increased the time spent in the target quadrant ($P < 0.05$; Fig. 6 c, f). There was no significant difference in swimming velocity among all three groups ($P > 0.05$, Fig. 6 g).

Rh- CXCL-12 treatment improved short-term neurological functions after SAH, and inhibition of CXCR4 and PI3K reversed such neuroprotective effects

The pretreatment of CXCR4 inhibitor, AMD3100, or PI3K inhibitor, LY294002, significantly reversed the neurobehavioral benefits of rh-CXCL-12 on the modified Garcia score and the beam balance score when compared with the corresponding control groups at 24 h after SAH (Fig. 7a, b).

Administration of rh-CXCL-12 attenuated neuroinflammation through the CXCR4/PI3K/Akt signaling pathway at 24 h after SAH.

Western blot results showed that the pathway-related proteins, CXCR4, PI3K, and p-Akt, and proinflammatory cytokines, IL-1 β , TNF- α , and IL-6, were upregulated in the SAH+vehicle group at 24 h after SAH when compared with the sham group (Fig. 8 a-h). Rh-CXCL-12 treatment further increased the expression levels of CXCR4, PI3K, and p-Akt, but decreased the expressions of pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α , compared with the SAH+vehicle group (Fig. 8 a-h). The administration of AMD3100 reversed such regulation of pathway-related proteins and the anti-neuroinflammatory effects of rh-CXCL-12 at 24 h after SAH (Fig. 8 a-h).

Moreover, without affecting the expression levels of CXCL-12 and CXCR4, pretreatment with selective PI3K inhibitor, LY294002, significantly suppressed the expression of PI3K and p-Akt, but increased protein levels of pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α at 24 h after SAH in the SAH+CXCL-12+LY294002 group compared with the SAH+CXCL-12+DMSO group (Fig. 9 a-h).

Administration of rh-CXCL-12 attenuated IL-1 β positive cells, which were abolished by either AMD3100 or LY294002 at 24 h after SAH.

The number of IL-1 β -positive cells were significantly increased in the SAH+vehicle group (Fig. 10 b-c). Rh-CXCL-12 treatment significantly attenuated IL-1 β secretion, which were reversed by CXCR4 or PI3K inhibitor (Fig. 10 b-c).

Discussion

The present study was the first to investigate the neuroprotective effects of CXCL-12 and explore the potential underlying mechanisms after experimental SAH in rats. Our results demonstrated that (1) endogenous protein levels of CXCL-12 and CXCR4 were increased, and peaked at 24 h after SAH. The CXCR4 receptors were expressed on microglia at 24 h after SAH. (2) Rh-CXCL-12 improved short- and long-term neurological deficits, and ameliorated brain edema at 24 h after SAH. Furthermore, rh-CXCL-12 treatment reduced the number of *M1 phenotype* activated *microglia in the* peri-hemorrhagic area in the ipsilateral cerebral cortex; (3) Administration of rh-CXCL-12 significantly increased the expression levels of CXCR4, PI3K, and p-Akt, but decreased the expression of pro-inflammatory cytokines, IL-1 β , TNF- α , and IL-6, and the number of IL-1 β -positive cells; (4) CXCR4 inhibitor, AMD3100, and PI3K inhibitor, LY294002, reversed the anti-neuroinflammatory effects of rh-CXCL-12 and its effects on the CXCR4/PI3K/Akt signaling pathway. Taken together, our results showed that the activation of CXCR4 with rh-CXCL-12 may exert a neuroprotective effect and improve neurological functions by reducing *M1 phenotype* activated microglia-mediated neuroinflammation after SAH, and these effects were, at least in part, via activation of the CXCR4/PI3K/Akt signaling pathway.

CXCR4, a chemokine receptor in the G protein-coupled receptor gene family, is widely found in the CNS and immune cells. It induces immune cell migration and nervous system development by binding to its ligand, CXCL-12. CXCR4 is involved in regulating the inflammatory response in central nervous system diseases, such as Alzheimer's disease [30], ischemic stroke [18], and Parkinson's disease [31]. In the present study, our results showed that the expression of exogenous CXCL-12 and CXCR4 increased at the early stage of SAH and peaked at 24 h after SAH. The increased expression of CXCL-12 and CXCR4 may explain its participation in the endogenous neuroprotective mechanisms after SAH, which were insufficient in overriding the injury.

Inflammatory mechanisms have been implicated in poor functional outcomes of EBI after SAH. Microglia, the resident immune cells in the brain, play a double-edged role in neuroinflammation, and are related to the functional outcome of SAH patients [32,33]. Microglia can be polarized into two different phenotypes after SAH, namely M1 and M2 [34]. M1-polarized microglia secrete numerous inflammatory cytokines, such as IL-1 β , IL-6, TNF- α , and inducible nitric oxide synthase, whereas M2-polarized microglia can produce neuroprotective properties, including transforming growth factor β and IL-10 [35]. In the early stage of brain injury after SAH, microglia are mainly activated as the M1 phenotype, which aggravates inflammatory responses [11]. In the current study, we found that CXCR4 co-localized with microglia using the double immunofluorescence method, and the number of CXCR4-positive microglia was significantly increased at 24 after SAH, which also indicated that CXCR4 was involved in the neuroinflammation in EBI after SAH.

We then evaluated the effects of rh-CXCL-12 in the experimental SAH model. Our results showed that the intranasal administration of rh-CXCL-12, at a dose of 15 μ g/kg, reduced brain water content, improved short-time neurobehavioral outcomes, which is used as the best dose of rh-CXCL-12 in further experiment.

Meanwhile, we demonstrated strong inflammatory responses and neutrophilic infiltration, as evidenced by increased M1 microglia/macrophage polarization (CD68-positive microglia) and MPO-positive cells, which was consistent with previous investigations [28]. Intranasal administration of rh-CXCL-12 reduced the number of M1 phenotype activated microglia and MPO-positive cells. In addition, neuronal apoptosis and the inflammatory response induced hippocampal neuronal degeneration, leading to the decline of memory and space learning ability after SAH [36]. In the current study, we found that rh-CXCL-12 notably improved long-term cognitive and memory impairment, which indicated that rh-CXCL-12 exerted a neuroprotective role in delayed brain injuries induced by SAH.

We further investigated the underlying molecular mechanism of CXCL-12-induced anti-inflammatory effects after SAH. The intracellular PI3K/Akt signaling pathway plays a key role in regulating the cell cycle, including cell proliferation, cellular quiescence, cancer, and longevity. Activation of PI3K results in the phosphorylation of Akt, which further participates in various molecular cascades. Phosphorylation of Akt maintains mitochondrial integrity and promotes cellular survival by resisting inflammation and oxidative stress-induced damage [28]. Many studies have found that the PI3K/Akt pathway is closely related to cerebral ischemic and hemorrhagic injury [36,37]. Sugawara et al. [38] found that intraperitoneal injection of simvastatin reduced nerve dysfunction and cerebral vasospasm in SAH rats, and intravenous injection of wortmannin (PI3K/Akt inhibitor) abolished the neuroprotective effects of simvastatin, which indicated that the PI3K/Akt signaling pathway is involved in the neuroprotective effect of cerebral vasospasm after SAH. Zhang HB et al. [39] demonstrated that propofol attenuates SAH-induced EBI by inhibiting inflammation and oxidative stress, which was reversed by LY294002 (PI3K/Akt inhibitor) administration. Furthermore, fibroblast growth factor-2 alleviated neurological impairments, brain edema, and neuronal apoptosis following SAH. PD173074 (PI3K/Akt inhibitor) abolished the anti-apoptotic effects of fibroblast growth factor-2 via suppression of the expression of PI3k [27]. These results indicate that PI3K/Akt signaling participates in anti-apoptosis and anti-neuroinflammation after SAH. Recent studies demonstrated that CXCL-12/CXCR4 could promote angiogenesis and regulate cell apoptosis by activating the PI3K/Akt signaling pathway, which indicated that the PI3K/Akt signaling pathway was a downstream molecule of the CXCL-12/CXCR4 axis [40,41]. In this study, we found that rh-CXCL-12 treatment significantly improved the modified Garcia and beam balance scores, and increased the expression levels of CXCR4, PI3K, and p-Akt, but decreased the expression of pro-inflammatory cytokines, IL-1 β , TNF- α , and IL-6, as well as IL-1 β -positive cells. Furthermore, CXCR4 inhibitor, AMD3100, and PI3K inhibitor, LY294002, reversed the neuroprotective effects of rh-CXCL-12 by decreasing the levels of IL-1 β , TNF- α , and IL-6. Taken together, rh-CXCL-12 attenuated neuroinflammation, suppressed M1-microglial polarization, and alleviated neutrophilic infiltration, which functioned, at least in part, by activating the CXCR4/PI3K/Akt signaling pathway after SAH.

This study has several limitations. First, this study focused primarily on the role of neuroinflammation in the EBI phase, and assessed the potential mechanisms of inflammatory responses at 24 h after SAH. However, neuroinflammation in delayed brain injury after SAH should also be investigated in future studies. Second, in addition to neuroinflammation, the pathogenesis of SAH includes neuronal apoptosis, oxidative stress, destruction of the blood-brain barrier, etc. However, the present experiment only focused

on neuroinflammation. Therefore, the observation and study of other mechanisms should be elucidated in future experiments. Third, CXCR4 has other downstream signaling pathways, such as MEK/ERK [42]. Therefore, more experiments are necessary to investigate the possible mechanisms of these signaling pathways and the neuroprotective effects of CXCL-12.

Conclusions

Our results demonstrated that the activation of CXCR4 with CXCL-12 improved short- and long-term neurological deficits, and attenuated neuroinflammation in EBI after SAH in rats. The protective effects of CXCL-12 were at least in part through activation of the CXCR4/PI3K/Akt signaling pathway. Therefore, early administration of CXCL-12 may provide a therapeutic strategy against brain injury after SAH.

Abbreviations

ANOVA: Analysis of variance; AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care International; BBB: blood-brain barrier; CNS: Central nervous system; CXCL-12: Cysteine-X-cysteine chemokine ligand 12; CXCR4: CXC chemokine receptor type 4; DMSO: Dimethyl sulfoxide; EBI: Early brain injury; i.n: Intranasally; i.p: Intraperitoneal; Iba-1: Ionized calcium binding adaptor molecule 1; IL-1 β : Interleukin-1 beta; MPO: Myeloperoxidase; SAH: Subarachnoid hemorrhage; SD: Standard deviation.

Declarations

Acknowledgements

Not applicable.

Ethical Approval and Consent to Participate

All animal experimental procedures were approved by the Guizhou Medical University and performed in accordance with the guidelines of the National Institutes of Health on the care and use of animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal quarters in Guizhou Medical University.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no conflict of interest.

Data availability statement

The data support the findings of this study and are available from the corresponding author upon reasonable request.

Authors' contributions

This study was designed by RG, LW, KJZ, GZ, and XH. The experiments were completed by RG, LW, GZ, XH, and GZ. XH, RG, and CL performed statistical analysis. XH, GZ, and CL finished writing the manuscript. GZ and KJZ provided supervision and final check. All the authors read the final version of this paper and approved it.

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Figures

Figure 1

Schematic summary. Rh-CXCL-12 attenuates neuroinflammation via the CXCR4/PI3K/Akt signaling pathway in a rat model of SAH.

Experiment 1: Time course of CXCL-12, CXCR4 and cellular localization of CXCR4 receptor

Groups:

Experiment 2: CXCL-12 attenuated short-term neuroinflammation and improved neurobehavioral function

Groups:

Experiment 3: CXCL-12 improved neurobehavioral outcomes after SAH

Groups:

Experiment 4: The effects of CXCR4/PI3K/AKT signaling pathway on neuroinflammation after SAH

Groups:

Figure 2

Experimental design. SAH, subarachnoid hemorrhage; WB, western blot; BWC, brain water content; IF, immunofluorescence; i.n, intranasal; i.p, intraperitoneal; DMSO, dimethyl sulfoxide.

Figure 3

Time course of CXCL-12 and CXCR4, as well as cellular localization of CXCR4 receptor after SAH. (a) Representative western blot bands of time course and densitometric quantification of CXCL-12 and CXCR4 (b-c) in the ipsilateral hemisphere after SAH. Data were presented as mean $\pm$ SD. * P < 0.05 vs sham group; n =6 per group. (d) Co-immunofluorescence staining of CXCR4 (red) with microglia (Iba-1, green) in the ipsilateral basal cortex at 24 h after SAH. Cell nuclei were counterstained with DAPI (blue). A small black square within the coronal section of the brain indicated the location of where the immunofluorescence staining images were taken. n =2 per group. Scale bar=100 μ m.

Figure 4

Intranasal administration of rh-CXCL-12 improved short-term neurological outcome and attenuated brain edema at 24 h after SAH. (a) Representative pictures of brain in sham and SAH groups (subarachnoid blood clots were mainly presented around the circle of Willis). (b) SAH grading scores of each group. (c-d) Intranasal administration of rh-CXCL-12 improved neurological performance on the modified Garcia and beam balance test at 24 h after SAH. (e) The quantification of brain water content in the left hemisphere, right hemisphere, cerebellum, and brain stem at 24 h after SAH. n=6 per group. Vehicle: sterile distilled water. Data were presented as mean $\pm$ SD. n=6 per group. *P<0.05 vs. Sham group; @P<0.05, @@P<0.01 vs. SAH+Vehicle group.

Figure 5

Intranasal administration of rh-CXCL-12 attenuated M1 phenotype microglia and neutrophil infiltration at 24 h after SAH. (a) Representative immunofluorescence staining of CD68-positive activated microglia and MPO-positive cells in the ipsilateral basal cortex at 24 h after SAH. Green indicated Iba-1- or MPO-positive staining, red indicated CD68-positive microglia staining, and blue indicated DAPI-positive nuclear staining. (b) A small black square within the coronal section of the brain indicated the location of where the immunofluorescence staining images were taken. (c) Quantitative analysis of CD68-positive activated microglia and (d) MPO-positive cells at 24 h after SAH. $n=4$ per group. Vehicle: sterile distilled water. Data were represented as mean $\pm$ SD. * $P<0.05$ vs. Sham group; @ $P<0.05$ vs. SAH+Vehicle group.

Figure 6

Rh-CXCL-12 improved long-term neurobehavioral outcomes after SAH. (a-b) The rotarod test of 5 and 10 rpm. (c) Representative heat map in probe test showed that rh-CXCL-12-treated SAH animals spent more time in probe quadrant. (d-e) Escape latency and swim distance of water maze test. (f) Probe quadrant duration. (g) Velocity of probe trial. $n=10$ per group. Data were presented as mean $\pm$ SD. * $P<0.05$ vs. sham group; @ $P<0.05$ vs. SAH + Vehicle group.

Figure 7

Effects of CXCR4 and PI3K inhibitor on neurobehavioral outcomes at 24 h after SAH. (a) Analysis of Modified Garcia score. (b) Analysis of Beam Balance score. n= 6 per group. Data were presented as mean $\pm$ SD. n=6 per group. Vehicle: sterile distilled water. DMSO, dimethyl sulfoxide. *P<0.05 vs. Sham group; @P<0.05 vs. SAH+Vehicle group.

Figure 8

Effects of CXCR4 inhibitor on downstream protein in proposed signaling pathway with rh-CXCL-12 treatment at 24 h after SAH. (a) Representative western blot bands of the proteins CXCL-12, CXCR4, PI3K, p-Akt, IL-1β, TNF-α, and IL-6, (b-h) Quantitative analysis of the relative expression level of these proteins at 24 h after SAH. *P < 0.05 vs. sham; @P < 0.05 vs. SAH+Vehicle group; &P < 0.05 vs. SAH+PBS group; n= 6 per group.

Figure 9

Effects of PI3K inhibitor on downstream protein in proposed signaling pathway with rh-CXCL-12 treatment at 24 h after SAH. (a) Representative western blot bands of the proteins, CXCL-12, CXCR4, PI3K, p-Akt, IL-1 β , TNF- α , and IL-6, (b-h) Quantitative analysis of the relative expression level of these proteins at 24 h after SAH. *P < 0.05 vs. sham; @P < 0.05 vs. SAH+Vehicle group; &P < 0.05 vs. SAH+ DMSO group; n= 6 per group.

Figure 10

Rh-CXCL-12 administration attenuated IL-1 β secretion, which were reversed by CXCR4 or PI3K inhibitor at 24 h after SAH. (a) A small black square within the coronal section of the brain indicated the location of where the immunofluorescence staining images were taken. (b) Quantitative analysis of IL-1 β -positive cells at 24 h after SAH. n=4 per group. Vehicle: sterile distilled water. Data were presented as mean $\pm$ SD. *P<0.05 vs. Sham group; @P<0.05 vs. SAH+Vehicle group; &P < 0.05 vs. SAH+ CXCL-12 group; #P < 0.05 vs. SAH+ DMSO group. (c) Representative immunofluorescence microphotographs of IL-1 β -positive cells. Red indicated IL-1 β -positive cell staining, and blue indicated DAPI-positive nuclear staining.

Supplementary Files

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