The Neuroprotective Effects of Necrostatin-1 on Subarachnoid Hemorrhage in Rats Are Possibly Mediated by Preventing Blood–Brain Barrier Disruption and RIP3-Mediated Necroptosis

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Abstract
Despite the substantial efforts to elucidate the role of early brain injury in subarachnoid hemorrhage (SAH), an effective pharmaceutical therapy for patients with SAH continues to be unavailable. This study aims to reveal the role of necroptosis after SAH, and explore whether the disruption of the blood–brain barrier (BBB) and RIP3-mediated necroptosis following SAH in a rat SAH model are altered by necrostatin-1 via its selective inhibition of receptor-interacting protein kinase 1 (RIP1). Sixty-five rats were used in the experiments. The SAH model was established using endovascular perforation. Necrostatin-1 was intracerebroventricularly injected 1 h before SAH induction. The neuroprotective effects of necrostatin-1 were evaluated with multiple methods such as magnetic resonance imaging (MRI) scanning, immunohistochemistry, propidium iodide (PI) labeling, and western blotting. Pretreatment with necrostatin-1 attenuated brain swelling and reduced the lesion volume on T2 sequence and ventricular volume on MRI 72 h after SAH induction. Albumin leakage and the degradation of tight junction proteins were also ameliorated by necrostatin-1 administration. In addition, necrostatin-1 decreased the number of PI-positive cells in the basal cortex, reduced the levels of the RIP3 and MLKL proteins, and inhibited the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Based on the findings from the present study, the selective RIP1 inhibitor necrostatin-1 functioned as a neuroprotective agent after SAH by attenuating brain swelling and BBB disruption. Moreover, the necrostatin-1 pretreatment prevented SAH-induced necroptosis by suppressing the activity of the RIP3/MLKL signaling pathway. These results will provide insights into new drugs and pharmacological targets to manage SAH, which are worth further study.

Keywords
subarachnoid hemorrhage, early brain injury, necroptosis, necrostatin-1, blood–brain barrier
Introduction

Subarachnoid hemorrhage (SAH), which is generally caused by the rupture of an intracranial aneurysm, is included in the category of fatal stroke. Patients who suffer an SAH inevitably have a high mortality rate, and delayed neurological deficits if they survive\(^1,2\). According to recent studies, early brain injury (EBI) is a major factor that worsens the outcome of SAH\(^3–5\). The possible mechanisms of EBI mainly include a sudden increase in the intracranial pressure coupled with a decrease in cerebral blood flow. This combination induces cell death, leading to a disruption of the blood–brain barrier (BBB) integrity and cerebral edema. Currently, an effective pharmaceutical therapy for patients with SAH is unavailable. Thus, novel pharmacotherapies or targets of treatment that ameliorate EBI in patients with SAH must be developed.

Programmed cell death (PCD), which plays an essential role in the pathological processes occurring after stroke, has become a hot topic in recent years. Three main types of PCD have been identified: apoptosis, autophagic cell death, and programmed necrosis. Programmed necrosis, which was also named necroptosis by Degterev et al.\(^6\), is a caspase-independent form of PCD that is mediated by pharmaceutical interventions. Currently, necroptosis has been reported in various models of brain injury, such as ischemic brain injury, intracranial hemorrhage, neurodegenerative disease, and traumatic brain injury (TBI)\(^6–10\). Degterev and colleagues also identified a small-molecule inhibitor of necroptosis named necrostatin-1\(^16\), which was characterized by its selective inhibitory effect on the receptor-interacting protein kinase 1 (RIP1)\(^11\). Numerous studies have recently discovered the neuroprotective effects of necrostatin-1. These beneficial effects include decreasing the brain injury volume, improving neurological functions, reducing production of reactive oxygen species, and exerting anti-neuroinflammatory, anti-endoplasmic reticulum stress, anti-apoptotic and anti-autophagic effects\(^12–16\). Meanwhile, several studies have explored the neuroprotective effects of necrostatin-1 at different time points after SAH, namely, at 24 h and 48 h\(^17–19\), and have indicated a role for necroptosis after SAH. Nevertheless, to our knowledge, the evidence that necroptosis occurs after SAH is scarce, and the potential mechanisms by which necrostatin-1 protects the BBB have not yet been identified. In addition, the effects of necrostatin-1 at 72 h or a later time point after SAH have not yet been elucidated.

Brain edema, BBB disruption, and neuroinflammation are the main components of central nervous system injury, and global edema is recognized to be one of the independent predictors of an unfavorable prognosis of SAH\(^19\). In fact, vasogenic edema is directly caused by BBB disruption\(^21,22\). Although hundreds of studies have examined necrostatin-1, its potential effects on attenuating brain edema by ameliorating BBB disruption have not been completely elucidated.

Taken together, we hypothesized that necroptosis might be a potential therapeutic target for SAH. In this article, we used a rat endovascular filament model and magnetic resonance imaging (MRI) scanning to determine whether necrostatin-1 alleviates brain edema, decreases ventricular and lesion volumes, ameliorates BBB disruption, or suppresses necroptosis within the first 72 h after SAH onset.

Materials and Methods

Animals and Study Design

Adult Sprague-Dawley rats weighing 300–320 g from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were used as animal models. Housing conditions of the rats were standardized by controlling the temperature and humidity control and maintaining a 12 h light/dark cycle. All animal experiments were approved by the Ethics Committee of Zhejiang University and the relevant procedures were performed in compliance to the NIH guidelines for the Care and Use of Laboratory Animals. Sixty-five rats were used in this study and segregated into the following groups: Sham group \((n = 14)\), SAH + vehicle group \((n = 26)\), and SAH + Nec-1 group \((n = 25)\). In the SAH + vehicle group, the endovascular perforation surgery was preceded by a vehicle pretreatment. The SAH + Nec-1 group was pretreated with Nec-1 before SAH surgery. The sham group underwent the same procedure as the SAH + vehicle group except for intracranial arterial perforation. Based on previous studies\(^8,13,23,24\) and our unpublished data regarding the time course of changes in the levels of the RIP3 and the mixed lineage kinase domain-like (MLKL) proteins after SAH, 72 h after SAH onset was chosen as the only endpoint throughout the experiment. We used six animals per group for MRI scanning, measurements of protein levels and matrix metalloproteases (MMP) activity; six animals per group were used for propidium iodide (PI) labeling, and another two animals per group were used for immunohistochemistry and immunofluorescence staining. The deceased animals that did not complete the indicated studies were removed from the groups and replaced with new, randomly selected rats to ensure the same number of animals in each group.

Experimental Induction of SAH

Two experienced investigators induced SAH in rats via endovascular perforation, according to a previous study\(^25\). Pentobarbital (40 mg/kg) was intraperitoneally injected to induce deep anesthesia in rats. Briefly, after dissection and distal transection of the external carotid artery (ECA), a blunted 4-0 monofilament nylon suture was advanced from the stump of the ECA into the internal carotid artery (ICA) until resistance was sensed. The suture was then inserted 2–3 mm further to penetrate the artery wall of the intracranial bifurcation of the ICA. In the sham operation, the surgery was terminated once resistance emerged.
Drug Administration

Necrostatin-1 was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in 1% DMSO in sterile saline. Three microliters of Nec-1 (1 μg) were administered 1 h before SAH induction via a left intracerebroventricular injection at an infusion rate of 0.5 μl/min. The dosage and timing of the necrostatin-1 pretreatment were based on previous studies. In the sham and SAH + vehicle groups, the same volume of vehicle was infused via the same route at the same time point. Details of the intracerebroventricular injection procedure are described in a previous study. After the induction of anesthesia via an intraperitoneal injection of pentobarbital (40 mg/kg), a small burr hole was created with a drill, and the needle of a 10 μl Hamilton syringe (Micro-liter701; Hamilton Company, Reno, NV, USA) was inserted at the following coordinates: 1.5 mm posterior, 1.0 mm lateral, and 3.5 mm depth relative to the bregma. The syringe was withdrawn 10 min after the injection, and the burr hole was sealed with bone wax.

Evaluation of the Neurological Score and SAH Grade

The neurological scores were graded using a previously reported protocol at different time points, namely, at 24, 48, and 72 h after SAH induction. The evaluation system was composed of six tests: spontaneous activity, symmetry in limb movement, forepaw outstretching, climbing, body proprioception, and the response to a touch of the vibrissae. Each test was scored as either 0–3 or 1–3 points, and the total scores ranged from 3 to 18 points. The behavioral tasks were administered to the animals in a random sequence. After the rats were euthanized, the SAH score was quantified using a previously described SAH grading system. The subarachnoid blood clots were observed over the basal cistern, which was divided into six parts based on its anatomy. Each segment received a score of 0 to 3 points, depending on the degree of bleeding, with the summary ranging from 0 to 18 points. All evaluations and grading processes were conducted by an independent observer, and the animals were evaluated in a random order. Animals with an SAH grade less than 6 were excluded from this study and replaced with new, randomly selected rats to ensure the same numbers of animals in each group.

MRI and Measurements

MRI was performed with a 3.0-T GE Discovery MR750 scanner (General Electric Company, Boston, MA, USA) 72 h after SAH. T2 fast spin-echo sequences were scanned in each rat using a field of view of 60 × 60 mm, a matrix of 256 × 256 and 9 coronal slices (2 mm thick). All the MRI data were analyzed by an independent researcher using NIH ImageJ software. Brain swelling was measured in images of nine sections as described in a previous publication, and the values were calculated as follows: ((volume of the left hemisphere – volume of the right hemisphere)/volume of the right hemisphere) × 100%. Ventricular volume was determined using the method reported in a previous study and was measured from the frontal horn of the lateral ventricle to the lateral aperture of the fourth ventricle using the formula A (A_n + A_n + 1) × d / 2, where A represents the ventricular area and d the distance between sections. The lesion volume on T2 sequences was confirmed as described in a previous study. Briefly, a pixel was considered abnormal if its value exceeded two standard deviations of the mean value of the contralateral hemisphere, and the result is presented as a ratio of the volume of the ipsilateral hemisphere.

Western Blot Analysis

Western blotting was conducted using our previously reported protocol. At 72 h after modeling, the rats were deeply anesthetized and transcardially perfused with PBS (0.1 mol/l, pH 7.4). The brains were removed, and the left basal cortices adjacent to blood clots were dissected, weighed, and homogenized. The samples were centrifuged at 1000 g for 10 min at 4°C. The supernatants were further centrifuged, and a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA) was used to measure the protein concentration. Equivalent amounts of samples (40 μg) were resuspended in loading buffer and heated at 100°C for 5 min. The samples and molecular markers were then loaded on the gel and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane. After blocking the nonspecific binding with 5% skim milk for 2 h, membranes were incubated overnight at 4°C on a shaker with the following primary antibodies: albumin (1:5000, Bethyl Laboratories, Montgomery, TX, USA Cat# A90-134A), ZO-1 (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat# SC-10804), Occludin (1:2000, Santa Cruz Biotechnology, Cat# SC-5562), Claudin-5 (1:800, Santa Cruz Biotechnology, Cat# SC-28670), MMP-9 (1:5000, Abcam, Cambridge, MA, USA, Cat# ab38898), RIP3 (1:1000, Novus Biologicals, Littleton, CO, USA, Cat# NBPI-77299), MLKL (1:500, Santa Cruz Biotechnology, Cat# SC-165025), caspase-8 (1:3000, Abcam, Cat# ab25901), IL-1β (1:800, Santa Cruz Biotechnology, Cat# SC-23459), IL-6 (1:2500, Abcam, Cat# ab9324), TNF-α (1:1000, Abcam, Cat# ab6671), and β-actin (1:5000, Abcam, Cat# ab8226). Next, membranes were incubated with the appropriate secondary antibodies (1:5000) for 1 h at room temperature. The bands were visualized using X-ray film and analyzed by densitometry using ImageJ software (NIH). The levels of target proteins were normalized to the levels of the structural protein to ensure comparability of the results between groups.
**Gelatin Zymography**

Gelatin zymography was performed using an MMP zymography assay kit (Applygen Technologies, Beijing, China, Cat# P1700) as described in a previous study. Equivalent amounts of the prepared protein samples (40 μg) were loaded and separated on a 10% Tris-glycine gel containing 0.1% gelatin as a substrate. After electrophoresis, the gels were placed in 2.5% Triton X-100 for 1 h to remove the SDS and then incubated at 37°C for 24 h with developing buffer. Then, the gels were stained with 0.5% Coomassie Blue R-250 for 2 h and then destained. The intensities of the proteolyzed bands indicated gelatinase activity. The intensity of zymography bands was measured and analyzed using Quantity One software (Bio-Rad).

**Propidium Iodide (PI) Labeling**

At 71 h after SAH induction, PI (Sigma-Aldrich, St. Louis, MO, USA) diluted to 10 mg/ml in sterile saline was injected intraperitoneally at a dose of 30 mg/kg. One hour later, the rats underwent transcardial perfusion with 0.1 mol/l PBS (pH 7.4) followed by 4% paraformaldehyde (pH 7.4). Then, the brains were harvested, fixed with 4% formaldehyde for 48 h at 4°C, and then dehydrated with a 30% sucrose solution until the brain samples sank to the bottom of the vessel (approximately 2–3 days). After fixation, the brains were embedded into tissue-freezing media and cut into coronal frozen sections (7 μm) at 150–200 μm intervals near the optic chiasma. PI-positive cells were counted in 200 × fields of the left basal cortex in three different sections per rat using a fluorescence microscope (Olympus, Tokyo, Japan). An independent observer counted the cells.

**Immunohistochemistry**

Immunohistochemistry was performed as described in a previous study. Rats were euthanized 72 h after SAH induction and transcardially perfused with 0.1 mol/l PBS (pH 7.4) followed by 4% paraformaldehyde (pH 7.4) as described above. Then, the brains were removed, fixed, dehydrated, and cut into coronal frozen sections (7 μm). The sections were incubated with a goat anti-mouse albumin antibody (1:500, Bethyl Laboratories, Cat# A90-134A) at 25°C for 2 h and washed with PBS (pH 7.4). After the sections were further incubated with secondary antibody for an additional 1 h at 25°C, the reaction product was visualized using a DAB peroxidase substrate kit SK-4100 (Vector Laboratories, Burlingame, CA, USA). An Olympus BX41 microscope was used to capture the images.

**Immunofluorescence Staining**

At 72 h after SAH induction, the rats were sacrificed and transcardially perfused with 0.1 mmol PBS (pH 7.4). Brain tissues were removed, fixed with 4% formaldehyde for 48 h at 4°C and then dehydrated with a 30% sucrose solution until the brain samples sank to the bottom of the vessel (approximately 2–3 days). After fixation and embedding in tissue-freezing media, the frozen brain tissues were cut into coronal sections (7 μm) at 150–200 μm intervals near the optic chiasma. The brain sections were preprocessed for 60 min in 0.01 mmol PBS (pH 7.4) containing 10% normal serum consistent with the species of the secondary antibody and 0.3% Triton X-100 to prevent nonspecific binding. Then, the brain sections were incubated overnight at 4°C with the following primary antibodies: Claudin-5 (1:100, Santa Cruz Biotechnology, Cat# SC-28670), RIP3 (1:250, Cell Signaling Technology, Danvers, MA, USA, Cat# 95702 s), and caspase-3 (1:200, Cell Signaling Technology, Cat# 9668s). After several washes with PBS, the sections were incubated with secondary antibodies for 2 h at 4°C in the dark. Then, the sections were rinsed and mounted onto slides with Fluoroshield™ containing DAPI (Sigma-Aldrich, Cat# F6057). Immunostaining was observed using a fluorescence microscope (Olympus).

**Statistical Analysis**

Western blot results from the three groups are described as the relative density of the target proteins normalized to β-actin and then compared with the sham group. The data are presented as the means ± SEM. Statistically significant differences between each pair of groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. A value of \( p < 0.05 \) was regarded as statistically significant. All statistical analyses were performed using SPSS (version 22.0 IBM, Armonk, NY, USA) and Prism (version 6.0) statistical software.

**Results**

**Neurological Scores, SAH Grade, and Mortality**

Representative images of brain samples from each group are presented in Fig. 1A. The induction of SAH significantly decreased the neurological scores compared with the sham group at 24, 48, and 72 h after SAH \( (p < 0.05 \) compared with the SAH + vehicle group, Fig. 1B). Pretreatment with Nec-1 significantly ameliorated the neurological deficits at each time point after SAH \( (p < 0.05 \) compared with the SAH + vehicle group, Fig. 1B). The statistical diagrams of detailed scores of each neurological test are shown in the supplementary file. SAH scores of the SAH + Nec-1 group were not significantly different from the SAH + vehicle group \( (p > 0.05 \), Fig. 1C). The mortality of each group was sham group 0% (0/14), SAH + vehicle group 46.2% (12/26) and SAH + Nec-1 group 44% (11/25). The pharmacological intervention in the SAH + Nec-1 group failed to significantly alter mortality \( (p > 0.05 \) compared with the SAH + vehicle group, Fig. 1D).
Necrostatin-1 Attenuated Brain Swelling, Reduced the Lesion Volume and Ventricular Volume on T2 Sequences after SAH

Representative MRI T2 images from each group are shown in Fig. 2A. Seventy-two hours after SAH, a remarkable increase in the brain swelling index was observed in the ipsilateral hemisphere compared with the sham group (p < 0.05, Fig. 2B). The necrostatin-1 pretreatment significantly attenuated the swelling of the left hemisphere at 24 h (p < 0.05 compared with the SAH + vehicle group, Fig. 2B). No lesions were observed on T2 images of the ipsilateral hemisphere in the sham group, while obvious lesions were noted in the SAH + vehicle group (Fig. 2A). Compared with the lesions in the SAH + vehicle group, the necrostatin-1 pretreatment significantly decreased the lesion volume caused by SAH on T2 sequences (p < 0.05, Fig. 2C). SAH induced a significant ventricular enlargement (p < 0.05 compared with the sham group, Fig. 2D), which was ameliorated by the application of Nec-1 (p < 0.05 compared with the SAH + vehicle group, Fig. 2D).

Necrostatin-1 Decreased Albumin Leakage after SAH

Immunohistochemistry and western blotting were performed to evaluate the BBB disruption. Representative images of albumin immunoreactivity in each group are presented in Fig. 3A. The onset of SAH significantly increased the leakage of albumin around the vessels at 72 h, whereas necrostatin-1 significantly decreased this process (p < 0.05 compared with the SAH + vehicle group, Fig. 3B).

Necrostatin-1 Prevented the Disruption of Tight Junction Proteins after SAH

The levels of occludin, claudin-5, and ZO-1 were detected and western blotting and immunofluorescence staining for claudin-5 was performed in each group to evaluate how the proteins constituting tight junctions were impacted by necrostatin-1 at 72 h after SAH. A significant decrease in the levels of these proteins was observed in the SAH + vehicle group, but not in sham group (p < 0.05, Fig. 4A–D). Necrostatin-1 significantly attenuated the degradation of occludin, claudin-5, and ZO-1 (p < 0.05 compared with the
SAH + vehicle, Fig. 4A–D). Immunofluorescence staining revealed high levels of claudin-5 in the sham group, but low levels in the SAH + vehicle group. The necrostatin-1 pre-treatment visibly improved claudin-5 expression in the basal cortex of the ipsilateral hemisphere (Fig. 4E). Based on these results, necrostatin-1 attenuated the disruption of BBB integrity at 72 h post-SAH.

**Necrostatin-1 Prevented the SAH-Induced Increase in MMP-9 Levels and Activity**

The impairment of the BBB caused by the degradation of tight junction components after stroke might be mediated by MMPs, particularly MMP-9. SAH induction significantly increased MMP-9 levels ($p < 0.05$ compared with the sham group, Fig. 5A and C), changes that were counteracted by the pretreatment with necrostatin-1 ($p < 0.05$ compared with the SAH + vehicle group, Fig. 5B and D). However, the proteolytic activity of MMP-2 did not change significantly among each group (Fig. 5B and E).

**Necrostatin-1 Decreased the Number of PI-Positive Cells after SAH**

With the aim of evaluating the effect of necrostatin-1 on protecting cells from injury, PI was used to identify the ruptured cells. The number of PI-positive cells was significantly increased in the basal cortex of the ipsilateral hemisphere at 72 h after SAH ($p < 0.05$ compared with the sham group, Fig. 6A and B), but the value was significantly decreased by the pretreatment with necrostatin-1 ($p < 0.05$ compared with the SAH + vehicle group, Fig. 6A and B).

**Necrostatin-1 Decreased the Activity of the RIP3/MLKL Signaling Pathway after SAH**

We performed double immunofluorescence staining for RIP3 and caspase-3 to distinguish cellular necroptosis from apoptosis. Some of the RIP3-positive cells were negative...
for caspase-3 (Fig. 7A). Levels of proteins involved in the RIP3/MLKL signaling pathway were also determined using western blotting to further confirm the role of necrostatin-1 in necroptosis. In the SAH + vehicle group, levels of the RIP3 and MLKL proteins were significantly increased compared with the sham group ($p < 0.05$, Fig. 7B, C and D, while necrostatin-1 administration exerted a significant inhibitory effect on the increased production of these proteins ($p < 0.05$ compared with the SAH + vehicle group, Fig. 7B, C and D). Caspase-8 suppressed the RIP1–RIP3 complex, and decreased caspase-8 levels can trigger necroptosis$^{40,41}$. Therefore, we also examined the level of the caspase-8 protein and observed a significant decrease 72 h after SAH ($p < 0.05$ compared with the sham group, Fig. 7B and E). The necrostatin-1 pretreatment generally increased caspase-8 levels ($p < 0.05$ compared with the SAH + vehicle group, Fig. 7B and E).

**Necrostatin-1 Attenuated Neuroinflammation after SAH**

Several pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, were also identified as contributors in SAH. Necrostatin-1 suppressed the SAH-induced increase in the levels of these pro-inflammatory cytokines at 72 h ($p < 0.05$ compared with the SAH + vehicle group, Fig. 8A-D).

**Discussion**

In the present study, the neuroprotective effect of necrostatin-1 on experimental SAH and its potential mechanisms were explored. The necrostatin-1 pretreatment significantly improved neurological functions, decreased brain swelling and the ventricular volume, reduced lesion volume on T2 images, and attenuated the BBB disruption. Necrostatin-1 also reduced the levels of pro-inflammatory cytokines and the number of injured cells. Furthermore, the activity of the RIP3/MLKL signaling pathway was also suppressed by pretreatment with necrostatin-1.

Necrostatin-1 is the first identified small-molecule inhibitor of necroptosis$^6$ that targets the RIP1 kinase in the necroptosis pathway$^{11}$. In addition, other necrostatins also target RIP1 through distinct mechanisms, such as necrostatin-3, 4, and 5. Although necrostatin-3 is less effective than necrostatin-1 in inhibiting the activity of wild-type RIP1, it still effectively inhibits the S161E mutant RIP1$^{11}$. Necrostatin-4 exhibits a slightly higher inhibitory constant (IC$_{50} = 0.37$ μM) than necrostatin-1 (IC$_{50} = 0.32$ μM)$^{42}$, but the half-maximum response (EC$_{50}$) for
Figure 4. Necrostatin-1 Prevented the Disruption of Tight Junction Proteins after SAH. (a) Representative Western blots showing levels of ZO-1, occludin and claudin-5 in the ipsilateral cortex in each group 72 h after SAH induction. (b–d) The relative band densities of ZO-1, occludin, and claudin-5. The densities of the protein bands were analyzed and normalized to β-actin, and compared with the mean value of the sham group. The bars represent the mean ± SEM. n = 6. *p < 0.05 versus sham, #p < 0.05 versus SAH + vehicle. (e) Representative microphotographs of immunofluorescence staining showing the levels of claudin-5 in each group. White arrows indicate the claudin-5-positive parts. Scale bar = 50 μm.
Figure 5. Effect of necrostatin-1 pretreatment on MMP-9 levels and activities 72 h after SAH induction. (a) Representative Western blots showing levels of MMP-9 in the ipsilateral cortex in each group 72 h after SAH induction. (b) Representative gelatin zymography bands showing activities of MMP-9 and MMP-2 in the ipsilateral cortex in each group 72 h after SAH induction. (c) The relative band densities of MMP-9. The densities of the protein bands were analyzed and normalized to β-actin, and compared with the mean value of the sham group. (d) The relative activity of MMP-9. (e) The relative activity of MMP-2. The bars represent the mean ± SEM. n = 6. *p < 0.05 versus sham, #p < 0.05 versus SAH + vehicle.

Figure 6. Effect of necrostatin-1 on cell injury in the ipsilateral basal cortex 72 h after SAH induction. (a) Representative microphotographs showed the co-localization of DAPI (blue) with PI (red) positive cells in the ipsilateral basal cortex at 72 h after SAH induction. White arrows indicate the PI-positive cells. (b) Quantitative analysis of PI-positive cells showed that necrostatin-1 could significantly reduce the percentage of PI-positive cells in the ipsilateral basal cortex at 72 h after SAH induction. The bars represent the mean ± SEM. n = 6. *p < 0.05 versus sham, #p < 0.05 versus SAH + vehicle. Scale bar = 50 μm.
necrostatin-5 (0.24 μM) exceeds the activity of necrostatin-1 (EC_{50} = 0.49 μM). In addition, another necroptosis inhibitor, necrostatin-7 (EC_{50} = 10.6 μM), does not inhibit RIP1 kinase. Therefore, necrostatin-1 is still the most widely used specific inhibitor of RIP1.

SAH is a fatal disease with few effective therapeutic strategies. The rodent model of SAH using a filament for endovascular perforation is a practical animal model to simulate the pathophysiological changes after aneurysmal SAH. Brain edema, which is a major component of EBI after SAH, is conducive to a series of pathological changes, such as brain swelling and neuronal cell death. As shown in our previous studies, strategies that decrease the brain water content improve the neurological scores of SAH rats. In the present study, we used a 3.0-T MRI to evaluate the brain swelling level instead of measuring the brain water content, because the former method is noninvasive and

Figure 7. Necrostatin-1 down-regulated RIP3/MLKL signaling pathway after SAH induction. (a) Representative microphotographs of immunofluorescence staining showing the localization of RIP3 (green) with caspase-3 (red) in ipsilateral basal cortex at 72 h after SAH induction. White arrows indicate the RIP3-positive and caspase-3 negative cells. (b) Representative Western blots showing levels of RIP3, MLKL, and cleaved caspase-8 in the ipsilateral cortex in each group 72 h after SAH induction. (c–e) The relative band densities of RIP3, MLKL, and cleaved caspase-8. The densities of the protein bands were analyzed and normalized to β-actin, and compared with the mean value of the sham group. The bars represent the mean ± SEM. n = 6. *p < 0.05 versus sham, #p < 0.05 versus SAH + vehicle. Scale bar = 50 μm.
could minimize animal deaths. The MRI scan also enabled us to measure the lesion volume on T2 sequences and the ventricular volumes of rats in each group. Lesion volume on T2 sequences are related to Garcia’s neurological score and the MRI grading of SAH.34 Another study examining SAH-induced hydrocephalus noted a correlation between the ventricular volume and the severity of SAH.33 In the current study, necrostatin-1 attenuated brain swelling, and reduced lesion volumes on T2 sequences and ventricular volumes after SAH. These findings were consistent with previous studies investigating the effects of necrostatin-1 on attenuating brain edema after ischemic brain injury23 and intracerebral hemorrhage.8 Since vasogenic brain edema is directly caused by BBB disruption, the effect of necrostatin-1 on the BBB disruption must be determined. Based on our findings, necrostatin-1 prevented albumin leakage, indicating that it might be effective in preventing BBB disruption. We further detected the levels of tight junction proteins to identify the mechanisms underlying these findings, and necrostatin-1 prevented their degradation. In addition, necrostatin-1 also decreased the level and activity of MMP-9, which is responsible for the degradation of tight junction proteins39 and correlates with functional outcomes after SAH.47 These results might partially explain the neuroprotective effect of necrostatin-1.

Necroptosis is a type of necrotic cell death that depends on RIP3.48 In this process, RIP1 interacts with RIP3 to form the necrosome.48,49 Therefore, the RIP1 inhibitor necrostatin-1 was considered a specific inhibitor of necroptosis. Recently, a pivotal role for MLKL in RIP3-mediated necroptosis has been illustrated in multiple studies.50,51 After phosphorylation by RIP3, MLKL forms an oligomer and directly disrupts membrane integrity.51 In fact, both apoptosis and autophagic cell death, along with their co-relationship, have already been acknowledged in SAH in our previous study.52 In the present study, necroptosis also

**Figure 8.** Necrostatin-1 attenuated neuroinflammation after SAH induction. (a) Representative western blots showing levels of IL-1β, IL-6, and TNF-α in the ipsilateral cortex in each group 72 h after SAH induction. (b–d) The relative band densities of IL-1β, IL-6, and TNF-α. The densities of the protein bands were analyzed and normalized to β-actin, and compared with the mean value of the sham group. The bars represent the mean ± SEM. n = 6. *p < 0.05 versus sham, #p < 0.05 versus SAH + vehicle.

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occurred after SAH, and necrostatin-1 has been reported to facilitate neuroprotection by ameliorating brain edema, BBB dysfunction, and neuroinflammation induced by SAH within the first 48 h\textsuperscript{17}. The levels of RIP3 and MLKL were both increased 72 h after SAH, and necrostatin-1 administration significantly decreased RIP3 and MLKL levels compared with the SAH + vehicle group, thus attenuating cell injury and improving neurological function. In addition, immunofluorescence staining showed that some of the RIP3-positive cells were negative for caspase-3. These results confirmed a role for necroptosis after SAH and supported the hypothesis that necrostatin-1 ameliorated this cell death pathway, similar to other types of experimental brain injury.

Neuroinflammation occupies an important position in the pathophysiological processes of distinct insults. The connection between brain injury after SAH and the production of a series of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, has been clarified by multiple studies\textsuperscript{53–56}. RIPs, basically, may also play a necroptosis-independent role in inflammation and mediate inflammasome activation through several pathways\textsuperscript{57}. Thus, we hypothesized that the RIP1 inhibitor necrostatin-1 might also exert an anti-inflammatory effect on EBI after SAH. The results of this study identified an effect of necrostatin-1 on preventing the upregulation of pro-inflammatory cytokines at 72 h after SAH. These results were consistent with previous studies of LPS-induced neuroinflammation\textsuperscript{24} and intracerebral hemorrhage models\textsuperscript{8}. In addition, inflammatory cytokines, particularly IL-1β, are considered the main activators of MMP-9\textsuperscript{58–60}, and this result also provides an explanation for the decreased levels and activity of MMP-9 observed after necrostatin-1 administration.

The blockage of cerebrospinal fluid (CSF) pathways by blood clots has been considered as the main cause of acute hydrocephalus. However, recent studies indicated that hydrocephalus may also have a relationship with inflammation\textsuperscript{61,62}, which could cause CSF hypersecretion by stimulating choroid plexus epithelium\textsuperscript{63}. As Nec-1 could not accelerate the absorption rate of the hematoma in subarachnoid cavity (Fig. 1C), a possible explanation of Nec-1’s effect of reducing ventricular volume is that it can control inflammation after SAH. However, the concrete mechanisms still need to be further explored.

In addition to necroptosis, several other types of PCD are believed to participate in EBI after SAH: apoptosis, autophagy, pyroptosis, and ferroptosis. Different types of cell death have been shown to simultaneously occur in neurons after SAH, resulting in a mixed morphologies of cell death\textsuperscript{64}. Among these pathways, apoptosis and autophagy have been identified as two major PCD forms. The crosstalk between autophagy and apoptosis after SAH has been mainly clarified in recent years, as autophagy protects cells from apoptosis via the mitochondrial pathway\textsuperscript{35,52}. At the same time, the relationship between necroptosis and apoptosis has also been explored by researchers. RIP1 and RIP3 contribute to both necroptosis and apoptosis, depending on the downstream effectors\textsuperscript{65,66}. This finding might explain the coexpression of RIP3 and caspase-3 in the images of double immunofluorescence staining shown in Fig. 6A. In addition, the autophagy protein p62 is also a necessary component of the RIP1/RIP3 necrosome and controls the switch between the cell death pathways apoptosis and necroptosis\textsuperscript{67}.

Our study investigated the neuroprotective effect of necrostatin-1 on experimental SAH and discussed the potential mechanisms. However, several limitations still exist in this study. The mechanism underlying the anti-inflammatory effect of necrostatin-1 was not completely elucidated. Based on another current study from our department\textsuperscript{18}, the regulation of NLRP3 inflammasome activation might be one possible explanation. Moreover, the long-term neuroprotective effects of necrostatin-1 require further research.

Conclusions

In summary, the selective RIP1 inhibitor necrostatin-1 functions as a neuroprotective agent after SAH by attenuating brain swelling and BBB disruption. In addition, the necrostatin-1 pretreatment prevents necroptosis after SAH by suppressing the activity of the RIP3/MLKL signaling pathway. These results provide insights into new drugs and pharmacological targets to manage SAH, and thus are worth further study.

Ethical Approval

This study was approved by the Ethics Committee of Zhejiang University, China.

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the Ethics Committee of Zhejiang University, China and were performed in compliance to the NIH guidelines for the Care and Use of Laboratory Animals.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclose the receipt of the financial support listed below for the research, authorship, and/or publication of this article: This study was supported by grants from Key Research and Development Project of Zhejiang Province (2018C03011), the National Science Foundation for Young Scientists of China (81700715), the Natural Science Foundation of Zhejiang Province (LQ15H090001, LY17H070001, LY17H090007, LY17H090008 and LY19H090009), the Project of Medicine and Health of Zhejiang Province (2014KYA093), the Project of Key Medical Subject and Innovation Platform Construction of Zhejiang Province (11CX08), the Project of Chinese Medicine Science and Technology of Department of Zhejiang Province (2017ZA086), the TCM...
Science and Technology Plan of Zhejiang province (2017ZZ013), and the Project of Science and Technology Plan of Hangzhou City (2014A62).

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Supplemental Material
Supplemental material for this article is available online.

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