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Research Paper

RIP1-RIP3-DRP1 pathway regulates NLRP3 inflammasome activation following subarachnoid hemorrhage

Keren Zhou [a,b,c], Ligen Shi [a,b,c], Zhen Wang [a,b,c], Jingyi Zhou [a,b,c], Anatol Manaenko [d], Cesar Reis [e], Sheng Chen [a,b,c,*], Jianmin Zhang [a,b,c,*]

[a] Department of Neurosurgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
[b] Brain research institute, Zhejiang University, Hangzhou, Zhejiang, China
[c] Collaborative Innovation Center for Brain Science, Zhejiang University, Hangzhou, Zhejiang, China
[d] Department of Neurology, University of Erlangen-Nuremberg, Erlangen, Germany
[e] Department of Physiology and Pharmacology, Loma Linda University, Loma Linda, CA, USA

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ABSTRACT

The NLRP3 inflammasome functions as a crucial component of the inflammatory response in early brain injury (EBI) after subarachnoid hemorrhage (SAH). However, the mechanisms underlying the activation of NLRP3 inflammasome has not been well elucidated. In this study, we hypothesized the RIP1-RIP3-DRP1 pathway was involved in the activation of the NLRP3 inflammasome following SAH. SAH was induced by endovascular perforation in rats. Necrostatin-1 (Nec-1) or mitochondrial division inhibitor (Mdivi-1) was administered 1 h after SAH by intraperitoneal injection. SAH grade, neurological function, brain water content, Western blot, ROS assay, immunofluorescence and transmission electron microscopy were performed. SAH led to the upregulation of RIP1, RIP3, phosphorylated DRP1 and NLRP3 inflammasome. Nec-1 treatment reduced RIP1, RIP3, phosphorylated DRP1 and NLRP3 inflammasome, subsequently alleviated brain edema and neurological deficits at 24 h following SAH. The treatment with Mdivi-1 inhibited the expression of DRP1 protein, attenuated mitochondria damage and the generation of ROS, inhibited NLRP3 inflammasome and ameliorated brain edema and neurological deficits at 24 h after SAH. The activation of the NLRP3 inflammasome in EBI after SAH was mediated by RIP1-RIP3-DRP1 pathway. Nec-1 and Mdivi-1 can inhibit inflammation and improve neurological function after SAH.

1. Introduction

Subarachnoid hemorrhage (SAH) is a severe cerebrovascular disease with high rate of mortality and disability (Etminan, 2015; van Gijn et al., 2007). The early brain injury (EBI), which occurs within first 72 h after SAH, has been considered a major cause of poor outcome in SAH patients (Caner et al., 2012; Sehba et al., 2012; Suzuki, 2015). The understanding of the molecular mechanisms underlying development of the EBI may improve the outcome of SAH patients. Recently, mounting evidence indicated that activation of NLRP3 inflammasome is a key component of post-SAH inflammatory response (Dong et al., 2016; Shao et al., 2016a). Previously, we demonstrated that P2X7R/NLRP3 inflammasome axis was involved in the pathogenesis of SAH. NLRP3 inflammasome expression significantly increased 24 h after SAH (Chen et al., 2013). However, the molecular mechanisms leading to the post-SAH NLRP3 inflammasome activation have not been well elucidated and the better understanding of them can lead to the development of new therapeutic approaches for SAH patients.

The receptor-interacting protein (RIP) is a serine-threonine protein kinase family, consisting of four isoforms RIP 1–4 (Zhang et al., 2009). The active form of RIP1 and RIP3 can constitute a stable formation of RIP1-RIP3 complex, which has been demonstrated to regulate programmed necrosis (Cho et al., 2009). Furthermore, there are indications that the RIP1-RIP3 complex is involved in the activation of inflammasome (Kang et al., 2013; Yabal et al., 2014; X. Wang et al. 2014). Necrostatin-1 (Nec-1) is a small molecule capable of inhibiting RIP1 kinase activity by preventing RIP1-RIP3 interaction. It inhibits necroptosis in various central nervous system (CNS) diseases, such as: ischemia, traumatic brain injury, and intracerebral hemorrhage (Northington et al., 2011; Su et al., 2015; You et al., 2008). However, the role of RIP1-RIP3 complex in the activation of NLRP3 inflammasome and the neuroprotective role of Nec-1 in inflammation after SAH have not been studied.

Reactive oxygen species (ROS), mainly associated with the malfunctioning mitochondria, played a significant role in the activation of NLRP3 inflammasome (Abais et al., 2015). Mitochondrial damage and fission are regulated by dynamin-related protein 1 (DRP1) (Tanaka and Youle, 2008). Mitochondrial division inhibitor (Mdivi-1), a selective inhibitor of DRP1, has been proved its neuroprotective effect in ischemia,

* Corresponding authors at: 88 Jiefang Road, Hangzhou, 310009, Zhejiang, China.
E-mail addresses: saintchan@zju.edu.cn (S. Chen), zjm135@zju.edu.cn (J. Zhang).

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traumatic brain injury and seizures (Wu et al., 2016; Xie et al., 2013; Zuo et al., 2014). In addition, infection with an RNA virus initiates assembly of the RIP1-RIP3 complex, which promotes activation of the GTPase DRP1 and its translocation to mitochondria to drive mitochondrial damage, production of ROS and eventually activation of the NLRP3 inflammasome (X. Wang et al. 2014). However, the anti-inflammatory effect of Mdivi-1 and the potential relation between RIP1-RIP3 and DRP1 have not been evaluated in EBI after SAH.

In the present study, we hypothesized the RIP1-RIP3-DRP1 pathway played a key role in regulating the activation of NLRP3 inflammasome in EBI after SAH. We first applied Nec-1 to investigate the role of RIP1-RIP3 in the phosphorylation of DRP1 and the activation of NLRP3 inflammasome after SAH. Second, we investigated the effect of DRP1 in regulating mitochondria ROS and activating NLRP3 inflammasome after SAH. At last, we studied whether Nec-1 and Mdivi-1 can attenuate brain edema and improve neurological function after SAH.

2. Methods

2.1. Animals and SAH model

Adult male Sprague–Dawley rats (300–340 g) were obtained from Animal Center of Zhejiang Chinese Medical University (Hangzhou, China). Rats were housed in a room with constant temperature (25 °C), humidity control and with a 12/12 h light/dark cycle, free access to food and water. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

SAH model was conducted by modified endovascular perforation method (Sugawara et al., 2008). Briefly, rats were anesthetized with pentobarbital injection (intraperitoneally 40 mg/kg). The left external and internal carotid artery were exposed and a 4.0 monofilament nylon suture was inserted into the left internal carotid artery through the external carotid artery stump until feeling resistance, and then advanced 3 mm to perforate the bifurcation of the anterior and middle cerebral artery. Sham rats underwent the same procedures except for the perforation. The inflammasome level reached the peak around 24 h after SAH (Chen et al., 2013). So, all the parameters were investigated 24 h after SAH induction.

2.2. Experimental design (Fig. 1)

In experiment 1, rats were randomly divided into 4 groups: the sham group ($n = 16$), SAH + vehicle group ($n = 16$), SAH + N(L) (low dose Nec-1, 3.5 mg/kg) group ($n = 14$), SAH + N(H) (high dose Nec-1, 10.5 mg/kg) group ($n = 16$). Postassessment included SAH grading, neurological score, brain water content, Western blot and immunofluorescence.

In experiment 2, rats were randomly divided into 4 groups: sham group ($n = 24$), SAH + vehicle group ($n = 24$), SAH + M(L) (low dose Mdivi-1, 1.2 mg/kg) group ($n = 14$), SAH + M(H) (high dose Mdivi-1, 3.6 mg/kg) group ($n = 24$). Postassessment included SAH grading, neurological score, brain water content, Western blot immunofluorescence. High dose Mdivi-1 group was used to detect ROS assay and electron microscopy.

2.3. Drug administration

Nec-1 was purchased from MedChem Express (Shanghai, CN) and was dissolved in DMSO (50 mg/ml) as stock. Before injection, it was diluted in PBS (Oerlemans et al., 2012). Nec-1 was administered in the SAH + N(L)(3.5 mg/kg) group and SAH + N(L)(10.5 mg/kg) group 1 h after surgery by intraperitoneal injection. The SAH + vehicle group received an equal volume of DMSO and PBS.

Mdivi-1 was purchased from MedChem Express (Shanghai, CN) and was dissolved in DMSO (50 mg/ml) as stock. It was diluted in sterile saline before injection (Zhao et al., 2014). Animals of SAH + M(L) and SAH + M(H) group were respectively given an intraperitoneal injection of Mdivi-1(1.2 mg/kg) and Mdivi-1(3.6 mg/kg) 1 h after SAH induction. Animals of SAH + vehicle group received an equal volume of DMSO and sterile saline in the same way and at the same time point.

2.4. Assessment of neurological score

The neurological status of all rats was evaluated at 24 h after SAH induction using the modified Garcia test (Sugawara et al., 2008). Briefly, the evaluation consists of six tests including: spontaneous activity (0–3), spontaneous movements of all limbs (0–3), movements of forelimbs (0–3), climbing wall of wire cage (1–3), reaction to touch on both side of trunk (1–3), and response to vibrissae touch (1–3). Possible scores ranged from 3 to 18. A lower score represents serious neurological deficits. The assessment of neurological score was performed by a partner who was blind to the experiment.

2.5. Measurement of SAH grade

The SAH grading score was used to estimate the degree of SAH as previously described (Sugawara et al., 2008). Briefly, the basal cistern was divided into six segments. Each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood clot: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid clots; grade 2, moderate subarachnoid clots with recognizable arteries; and grade 3, blood clots covering all arteries. A total score ranging from 0 to 18 was obtained by adding the scores of six segments. The grading of SAH was performed by a partner who was blind to the experiment. Rats with the SAH grade lower than 9 were excluded from this study.

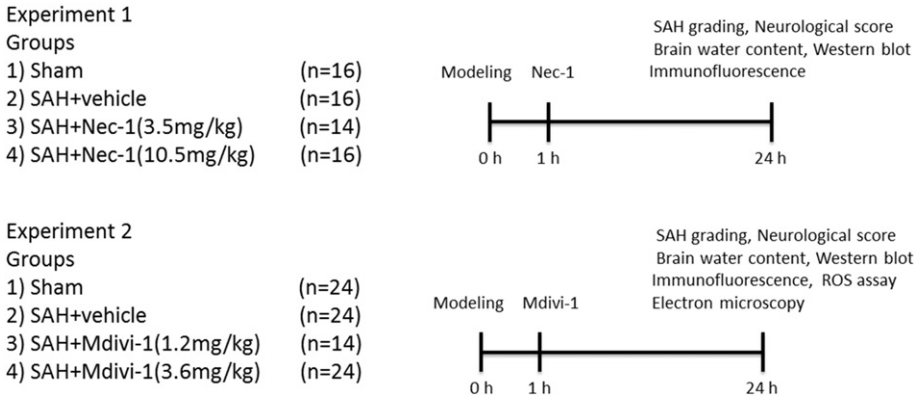

Fig. 1. Experimental designs and animal group classification.