International Immunopharmacology 40 (2016) 492–500

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The neuroprotection of Sinomenine against ischemic stroke in mice by suppressing NLRP3 inflammasome via AMPK signaling

Jing Qiu [a,1], Min Wang [c,1], Jun Zhang [b,d,1], Qing Cai [b], Dan Lu [b], Yansong Li [c], Yushu Dong [a], Tianzhi Zhao [b,*], Huisheng Chen [a,*]

[a] Department of Neurology, The General Hospital of Shenyang Military Region, Shenyang, Liaoning 110016, PR China
[b] Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710038, PR China
[c] Department of Neurology, The 463rd Hospital of PLA, Shenyang, Liaoning 110042, PR China
[d] Department of Neurosurgery, Dalang Hospital, Dongguan, Guangzhou 523000, PR China

ARTICLE INFO

Article history:
Received 8 July 2016
Received in revised form 22 September 2016
Accepted 24 September 2016
Available online xxxx

Keywords:
Ischemic stroke
Secondary brain injury
Sinomenine
NLRP3 inflammasome
AMPK
Glia

ABSTRACT

Neuroinflammation remains the primary cause of morbidity and mortality in stroke-induced secondary brain injury. The NOD-like receptor pyrin 3 (NLRP3) inflammasome is involved in diverse inflammatory diseases, including cerebral ischemia, and is thus considered an effective therapeutic target. In the present study, we investigated the neuroprotection of Sinomenine (SINO), a potent natural anti-apoptotic and anti-inflammatory molecule, against cerebral ischemia in a mouse model of middle cerebral artery occlusion (MCAO) in vivo and in an oxygen glucose deprivation (OGD)-treated astrocytes/microglia model in vitro. SINO administration intraperitoneally alleviated the cerebral infarction, brain edema, neuronal apoptosis, and neurological deficiency after MCAO induction. SINO also attenuated astrocytic and microglial activation in the ischemic hemisphere. NLRP3 inflammasome activation after MCAO and OGD induction, with the up-regulation of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), cleaved caspase-1 and pro-inflammatory cytokines, was significantly inhibited by SINO treatment both in vivo and in vitro. In addition, SINO reversed the OGD-induced inhibition of AMPK phosphorylation in vitro. Further, the suppressive effect of SINO on NLRP3 inflammasomes was blocked by an AMPK inhibitor, Compound C. Our findings demonstrate that SINO exerts a neuroprotective effect in ischemic stroke by inhibiting NLRP3 inflammasomes via the AMPK pathway, which also provides evidence of a novel treatment for clinical stroke therapy.

1. Introduction

Ischemic stroke leads an important cause of disability and mortality in adults worldwide and accounts for approximately 100,000 deaths annually [1,2]. Unfortunately, only 3 to 5% of such patients can receive the efficacious treatments of thrombolysis or mechanical embolus removal in cerebral ischemia (MERCI) [3,4] because acute neuronal damage occurs immediately (i.e., within several hours) following the arterial embolism. Thus, the attenuation of secondary brain injury after ischemia is important and susceptible in clinical treatment [5]. Multiple cellular pathological processes, including oxidative stress, excitotoxicity, inflammation, and mitochondrial dysfunction, have been demonstrated to be involved in cerebral ischemic injury [6–8]. Accumulating evidence suggests that neuroinflammation plays essential roles in secondary brain injury after ischemic stroke [9,10]. However, the detailed mechanism of inflammatory modulation after ischemic stroke is unclear.

The inflammasomes play critical roles in the initiation of inflammation and the development of innate immune responses in the central nervous system (CNS) [11–13]. The NLRP3 inflammasome, which consists of the NLRP3 scaffold, the ASC (PYCARD) adaptor, and caspase-1, is currently the most fully characterized inflammasome [12]. Activation of the NLRP3 inflammasome promotes the release of several pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-18, and TNF-α [12,14]. Recent evidence has shown that NLRP3 inflammasome activation is involved in diverse neurological and neurodegenerative diseases, including ischemic/hemorrhagic stroke [15,16], traumatic brain injury (TBI) [17], Parkinson's disease (PD) [18], and amyotrophic lateral sclerosis (ALS) [19]. Therefore, intervention of the NLRP3 inflammasome to suppress the excessive inflammatory response may provide a novel therapeutic strategy for cerebral ischemia.

Sinomenine, an alkaloid extracted from the medicinal plant Sinomenium acutum, was proven to possess anti-inflammatory effects and was used for a long time in the treatment of neuralgia and rheumatic diseases in China [20]. Extensive pharmacological and clinical studies

* Corresponding authors.
E-mail addresses: zhaotianzhi1981@163.com (T. Zhao), chsshenyang@sina.cn (H. Chen).
[1] Jing Qiu, Min Wang and Jun. Zhang contributed equally to this work.

http://dx.doi.org/10.1016/j.intimp.2016.09.024
1567-5769/

on SINO primarily focused on the immune, cancer, cardiovascular, and nervous systems [21–23]. SINO might regulate immune reactions by inhibiting the activation of macrophages, peripheral blood monocytes, and microglia [24,25] and by reducing the secretion of prostaglandin E3, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 [20,26].

However, little is currently known about SINO in the treatment of ischemic stroke. In this study, based on the in vivo and in vitro model, we sought to examine whether SINO possessed neuroprotective effects and to explore the underlying mechanisms of SINO regulating NLRP3 inflammasomes after cerebral ischemia.

2. Experimental procedures

2.1. Animal preparation and MCAO mice model

Adult male *C57BL/6* mice (20–25 g, 8–12 weeks old) were used and supplied by the Laboratory Animal Center of the Fourth Military Medical University in the present study. All procedures and protocols were approved by the Committee for Experimental Animal Use and Care of the Fourth Military Medical University (China). The greatest efforts were made to minimize the number of animals used and their suffering. All animals were fed freely with regular chow and water, and were housed in a temperature-controlled room with a 12 h light-dark cycle.

The middle cerebral artery occlusion (MCAO) surgery was performed as previously described [27]. Briefly, the mice were anesthetized with 1.5–2% isoflurane mixed with oxygen and nitrogen. A 6-0 rounded tip nylon suture was gently advanced from the common carotid artery to the internal carotid artery and wedged into the circle of Willis to obstruct the origin of the middle cerebral artery (MCA). The filament was left for obstruction for 60 min and then withdrawn for the reperfusion. Transcranial laser Dppler flowmetry was used to confirm the successful MCAO (a criterion of <20% of baseline blood flow remaining after filament placement) and reperfusion (>85% of baseline) procedures in mice [28]. The sham group was induced by an identical operation, except there was no occlusion of the MCA. A constant-temperature blanket was used to keep the body temperature at 37 ± 1 °C until the animals recovered from surgery.

2.2. Drug administration and animal groups

Sinomenine (purchased from Selleck) was injected intraperitoneally in mice at the doses of 10 or 20 mg/kg daily for 3 days after MCAO surgery (the first injection was administered 30 min after operation). The vehicle group received the same volume of saline. Mice were randomly divided into four groups (n = 8 for each group in each experiment): sham group; MCAO group treated with vehicle; and MCAO group treated with 10 or 20 mg/kg SINO daily for 3 days.

2.3. Neurological deficiency evaluation

Neurological deficiency was evaluated 3 days after MCAO according to a 4-tiered grading system (0, no observable deficits; 1, torso flexion to the right; 2, spontaneous circling to the right; 3, leaning/falling to the right; 4, no spontaneous movement), a 21-point Garcia test score system (including 7 individual tests: spontaneous activity (I), axial sensation (II), vibrissae proprioception (III), symmetry of limb movement (IV), lateral turning (V), forelimb outstretching (VI) and climbing (VII); each test received a score between 0 (worst) and 3 (best) and the total score was out of 21 points (maximum)) and a 10-point score system of forelimb motor test (expressed as the number of successful paw placements out of 10 consecutive vibrissae-elicited excitation) as reported previously [29,30]. The trained investigators were blinded to the animal groups in the test.

2.4. Infarct volume measurement

The animals were anesthetized, and the brains were carefully harvested 3 days post-MCAO. Coronal brain slices were cut with a rocking microtome and collected in pre-warmed 2% 2,3,5-triphenyltetrazoliumchloride (TTC) (Sigma) for 10 min, followed 30 min of fixation with 4% paraformaldehyde in PBS (pH = 7.4). The unstained area of the brain slice was defined as infarction, and the photographed images were analyzed using the NIH Image J software to determine the infarct volume as described previously [31]: [1 − (total ipsilateral hemisphere-infarct) / total contralateral hemisphere] × 100%.

2.5. Brain water content measurement

The mice were deeply anesthetized and decapitated 3 days after MCAO. Brain edema was analyzed according to the method described previously [32]. Briefly, the wet brains were weighed and then immediately dried at 95 °C overnight. The brain water content was calculated as follows: [(wet tissue weight − dry tissue weight) / wet tissue weight] × 100%.

2.6. Primary mixed glial cell culture

Primary mixed glial cells were prepared from the cortex of mice at P0 according to a protocol that was reported previously [33]. The cortex tissues were trypsinized and plated at a density of 5×10^7 cells per 75 cm^2 flask in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium containing 10% fetal bovine serum (FBS) (Gibco, NY, USA). The culture medium was changed subsequently twice a week, and the samples were cultured for two weeks before the following in vitro procedure.

2.7. Oxygen glucose deprivation (OGD) management and treatment

The OGD cell model was used to mimic the ischemic stroke mice model. OGD treatment is a mature and widely used in vitro model in the study of cerebral ischemic stroke. The OGD procedures were followed by the protocol described previously [34]. Primary mixed glial cells were cultured with glucose/FBS-free DMEM (GIBCO, CA, USA) in an incubator (Thermo scientific) with a premixed gas (95% N_2, 5% CO_2) for 5 h. Then, the culture medium was renewed with normal DMEM (10% FBS) and normal gas (95% air and 5% CO_2). The following concentrations of SINO were added: 0.1, 0.5, 1.0 mM SINO, along with 10, 20, 40 μM compound C (CC, an efficient and specific AMPK inhibitor), respectively, for 12 h.

2.8. Histological staining

Histological assessment was performed according to a frozen-section staining protocol described previously [35,36]. Briefly, the brain samples were fixed with 4% paraformaldehyde in PBS (pH = 7.4) and then were cryoprotected for 24 h at 4 °C in 30% sucrose. Coronal sections (25 μm) were cut in a cryostat microtome and collected for immunofluorescence and Nissl staining. The primary antibodies were anti-glial fibrillary acidic protein (GFAP) (1:4000, Millipore) and anti-IBa1 (1:400, Abcam), and the secondary antibodies were donkey anti-mouse IgG (Alexa Fluor 594, 1:400, Abcam) and donkey anti-rabbit IgG (Alexa Fluor 488, 1:400, Abcam).

2.9. Western blotting

The sham and ischemic mice were sacrificed 3 days after surgery, and the ischemic penumbra was collected for immunoblotting. Total 20 μg of protein that had been quantified using a BCA protein assay was electrophoresed and electrotransferred to PVDF membranes, which were then incubated with primary antibodies at 4 °C overnight: