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Nafamostat mesilate improves function recovery after stroke by inhibiting neuroinflammation in rats

Chenhui Li [a], Jing Wang [a], Yinquan Fang [a], Yuan Liu [a], Tao Chen [a], Hao Sun [a], Xin-Fu Zhou [b,*], Hong Liao [a,*]

[a] Jiangsu Key laboratory of Drug Screening, China Pharmaceutical University, 24 Tongjiaxiang Street, Nanjing 210009, China
[b] School of Pharmacology and Medical Sciences, University of South Australia, Adelaide, SA 5000, Australia

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ABSTRACT

Inflammation plays an important role in stroke pathology, making it a promising target for stroke intervention. Nafamostat mesilate (NM), a wide-spectrum serine protease inhibitor, is commonly used for treating inflammatory diseases, such as pancreatitis. However, its effect on neuroinflammation after stroke was unknown. Hence, the effects of NM on the inflammatory response post stroke were characterized. After transient middle cerebral artery occlusion (tMCAO) in rats, NM reduced the infarct size, improved behavioral functions, decreased the expression of proinflammatory mediators (TNF-α, IL-1β, iNOS and COX-2) in a time-dependent manner and promoted the expression of different anti-inflammatory factors (CD206, TGF-β, IL-10 and IL-4) at different time points. Furthermore, NM could inhibit the expression of proinflammatory mediators and promote anti-inflammatory mediators expression in rat primary microglia following exposure to thrombin combined with oxygen–glucose deprivation (OGD). The immune-modulatory effect of NM might be partly due to its inhibition of the NF-κB signaling pathway and inflammasome activation after tMCAO. In addition, NM significantly inhibited the infiltration of macrophage, neutrophil and T lymphocytes, which was partly mediated by the inhibition of monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Taken together, our results indicated that NM can provide long-term protection of the brain against tMCAO by modulating a broad components of the inflammatory response.
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1. Introduction

Ischemic brain damage is mediated by a complex series of biochemical and molecular mechanisms. Among these mechanisms, there is increasing evidence showing that inflammation is one of the key elements in the pathobiology of ischemic stroke (Chamorro et al., 2012; Fu et al., 2015). The inflammatory response following stroke is characterized by the activation of microglia and the infiltration of circulating inflammatory cells (Jin et al., 2010), which may progress over hours to days after stroke, and have extensive influence on stroke pathology. Microglia, for example, can be either neuroprotective or neurotoxic, and recent studies have showed that promoting the anti-inflammatory activation of microglia may provide long-term protection in experimental stroke (Jin et al., 2014).

Recently, a novel concept of 'thrombo-inflammation' has been raised for ischemic stroke, which describes a close relationship between thrombotic factors and the inflammatory response (Gob et al., 2015; Nieswandt et al., 2011). The serine proteases, such as thrombin, factor Xa (FXa) and kallikrein are best known for their functions on thrombosis (Siller-Matula et al., 2011). Besides, they may also play crucial roles in inflammation. For example, thrombin promotes the activation of NOD-like receptors family, pyrin domain-containing 3 (NLRP3) inflammasome in macrophage (Rossol et al., 2012) and microglia proinflammatory activation (Lee et al., 2005). FXa and kallikrein have also been reported to be links between inflammation and thrombosis (Prassas et al., 2015; Zuo et al., 2015). It is becoming increasingly apparent that thrombin, FXa and kallikrein are increased after ischemic stroke (Thevenet et al., 2009; Chen et al., 2012; Gob et al., 2015), and due to their dual role in thrombosis and inflammation, they may be promising targets for stroke treatment.

Nafamostat mesilate (NM), a wide-spectrum serine protease inhibitor, which inhibits several serine proteases such as thrombin, plasma kallikrein and FXa, is capable of blocking a battery of components in the coagulation system and the inflammatory cascades. For example, NM inhibits the inflammatory response in intestine, heart ischemia (Gobbetti et al., 2012; Schwertz et al., 2008) and multiple

* Corresponding authors.
E-mail addresses: xin-fu.zhou@unisa.edu.au (X.-F. Zhou), hliao@cpu.edu.cn (H. Liao).

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sclerosis (Li et al., 2009), and our previous research has demonstrated that NM attenuates neuronal damage after stroke through thrombin inhibition (Chen et al., 2014). Therefore, NM might be a potential candidate to reduce 'thrombo-inflammation' in stroke.

In this study we explored the effects of NM on behavioral recovery and neuroinflammation post transient middle cerebral artery occlusion (tMCAO) in rats. We demonstrated that NM improves behavioral recovery after tMCAO in rats by inhibiting the expression of proinflammatory mediators in a time-dependent manner, promoting the expression of different anti-inflammatory mediators at different time points, and inhibiting the recruitment of circulating immunocyte, which might be partly via inhibiting the activation of NF-κB signaling pathway and NLRP3 inflammasome. In addition, an *in vitro* hypoxia model, in which cells were exposed to oxygen–glucose deprivation (OGD) in the presence of thrombin, was taken to explore the mechanism of NM on neuroinflammation.

2. Materials and methods

2.1. Animals and surgery

Adult male Sprague–Dawley (SD) rats weighing 260–280 g were obtained from Zhejiang Laboratory Animals Center (Hangzhou, China) and housed in controlled-temperature environment under a 12-h light/dark cycle and allowed free access to food and water. All animal handling and surgical procedures were approved by the Animal Research Ethics Committee of China Pharmaceutical University. tMCAO was performed as reported earlier with some modifications (Longa et al., 1989). Briefly, rats were initially anesthetized with 3% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) in 0.9% saline (Sinopharm Chemical Reagent Co., Ltd). The body temperatures were maintained at 37.0 °C with warming pads, and the cerebral blood flow (CBF) was monitored via laser Doppler flowmetry (Moor Instruments, Essex, UK). The bifurcation of the right common carotid artery was exposed, and a 3-0 poly-lysine (Sigma–Aldrich, USA) coated monofilament nylon suture was advanced through external carotid artery into the lumen of internal carotid artery to occlude the origin of the middle cerebral artery (MCA). In the ischemia phase, the blood perfusion dropped >75% of the base line was considered as successful ischemia. Rats were re-anesthetized and the suture was gently withdrawn to restore the blood flow after 2 h occlusion. And blood gases were monitored with i-STAT Portable Clinical Analyzer (Abbott, Ontario, Canada), blood pressure were monitored with biological function experiment system (Zhenghua, Anhui, China), and temperature were monitored with thermometer. These physiological factors monitored before, during, and after surgery.

2.2. Drug treatment

The specific thrombin inhibitor argatroban (Enzo Life Sciences, USA) was used as positive control. NM (Nanjing D&R Pharmaceutical Company, Nanjing, China) and argatroban were diluted in 5% glucose (Sinopharm Chemical Reagent Co., Ltd). Animals were randomly divided into six groups, including sham, vehicle, argatroban (3.4 mg/kg) and NM (0.01, 0.1 and 1 mg/kg) groups, respectively. After tMCAO surgery, rats in the vehicle group and drug-treated groups were intravenously treated with glucose or drugs respectively at the beginning of ischemia (0 h) and the moment of reperfusion (2 h). And drugs were administered again with the same time interval at 4 h and 6 h after ischemia onset.

2.3. Behavioral tests

Behavioral tests were performed at different days after MCA occlusion.

1) Corner test: the corner test was used to assess the sensorimotor deficit as described before with some modification (Jin et al., 2014). Briefly, a rat was placed between two boards with an angle of 30° and facing the corner. Both sides of the vibrissae were stimulated together when the rat entered deep into the corner. The rat then turn back to face the open side. The non-ischemic rats turned non-selectively left or right, but the tMCAO treated rats preferentially turned toward the non-impaired side. Between each trails, there was a rest period of 10 s. The turns toward the non-impaired side were recorded from ten trials for each test.
2) Grip-traction test: the modified grip-traction test was used to test the muscle strength of the rat by hanging the rat to a horizontal rope (4 mm in diameter) by its forepaws (Bona et al., 1997). Time to falling (maximum 60 s) was recorded.
3) Beam balance test: the beam was 2.5 cm in width, 80 cm in length, and 60 cm in height. The beam balance test was performed as described before (Chen et al., 2001).
4) Limb-placing test: the test was taken to test the asymmetry of the upper limb movement (Bona et al., 1997). This test consist six limb-placing tasks, with a 3-point scale each.
5) Y-maze test: the test was performed as descried previously (Tang et al., 2013). The Y-maze was constructed of black plastic walls (10 cm high), consisting of three compartments (10 cm × 10 cm) connected with passages (4 cm × 5 cm), with the floor of 3.175 mm stainless steel rods (8 mm apart). On day 1 (the training trial), each rat was placed in the conjunction area of the maze and allowed to explore the maze freely for 5 min with no electric shocks. Then two of the three arms were turned to be shocks-available but light-off, with the third one was shock-free and light-on. Each rat was trained for 10 times, the training was completed once the rat entered the shock-free arm and stayed for 30 s, which was taken as right choice. The testing trail was taken on the next day, each rat was tested for 10 times following the same procedures as on day 1. The times and the latency to enter the shock-free arm were recorded.
6) Longa test: this test was assessed using a 5-point scale as described previously (Chen et al., 2014): 0, no observable deficits; 1, failure to extend the left forepaw; 2, circling to the left; 3, falling to the left; and 4, unable to move spontaneously.

2.4. Histological assessments of the brain damage following MCAO

At 7 days after MCAO, the infarct volumes were evaluated with triphenyl-2,3,4-tetrazolium-chloride (TTC) stain. The brains were coronal cut into 2-mm-thick slices and stained with saline containing 2% TTC (Wako Pure Chemical Industries, Ltd., Osaka) at 37 °C for 10 min. The infarct areas were measured using Image J (NIH). The infarction rates were calculated as the infarction volume/the brain volume × 100%.

2.5. Western blotting

At 6 or 24 h after tMCAO, tissues collected from the MCA perfusion area (coronal between +3 and −2 mm relative to Bregma in the ipsilateral cortex) were homogenized with radio immunoprecipitation assay (RIPA) buffer (Beyotime, Hangzhou, China) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein concentrations were measured with bicinchoninic acid assay (BCA) kit (Beyotime). Equal amounts of protein (50 μg) of each samples was separated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to nitrocellulose filter membrane. Membranes were blocked with 2.5% (w/v)

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