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Full-length Article

Recombinant Netrin-1 binding UNC5B receptor attenuates neuroinflammation and brain injury via PPARγ/NFκB signaling pathway after subarachnoid hemorrhage in rats

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ABSTRACT

Neuroinflammation is an essential mechanism involved in the pathogenesis of subarachnoid hemorrhage (SAH)-induced brain injury. Recently, Netrin-1 (NTN-1) is well established to exert anti-inflammatory property in non-nervous system diseases through inhibiting infiltration of neutrophil. The present study was designed to investigate the effects of NTN-1 on neuroinflammation, and the potential mechanism in a rat model of SAH. Two hundred and ninety-four male Sprague Dawley rats (weight 280–330 g) were subjected to the endovascular perforation model of SAH. Recombinant human NTN-1 (rh-NTN-1) was administered intravenously. Small interfering RNA (siRNA) of NTN-1 and UNC5B, and a selective PPARγ antagonist bisphenol A diglycidyl ether (BADGE) were applied. Post-SAH evaluations included neurobehavioral function, brain water content, Western blot analysis, and immunohistochemistry. Our results showed that endogenous NTN-1 and its receptor UNC5B level were increased after SAH. Administration of rh-NTN-1 reduced brain edema, ameliorated neurological impairments, and suppressed microglia activation after SAH, which were concomitant with PPARγ activation, inhibition of NFκB, and decrease in TNF-α, IL-6, and ICAM-1, as well as myeloperoxidase (MPO). Knockdown of endogenous NTN-1 increased expression of pro-inflammatory mediators and MPO, and aggravated neuroinflammation and brain edema. Moreover, knockdown of UNC5B using specific siRNA and inhibition of PPARγ with BADGE blocked the protective effects of rh-NTN-1. In conclusion, our findings indicated that exogenous rh-NTN-1 treatment attenuated neuroinflammation and neurological impairments through inhibiting microglia activation after SAH in rats, which is possibly mediated by UNC5B/PPARγ/NFκB signaling pathway. Exogenous NTN-1 may be a novel therapeutic agent to ameliorating early brain injury via its anti-inflammation effect.

1. Introduction

Activation of neuroinflammatory response plays a crucial role in the progression and exacerbation of brain injury following subarachnoid hemorrhage (SAH) (Lucke-Wold et al., 2016). The innate immune response triggered by SAH is characterized by leukocyte infiltration, resident immune cells activation, and release of pro-inflammatory mediators within brain tissues, which result in blood-brain barrier (BBB) disruption and brain injury via various mechanisms (Frontera et al., 2012; Frontera et al., 2017). Therefore, inhibition of inflammatory response represent a potential treatment for attenuating brain injury following SAH (Chen et al., 2014; Provencio et al., 2016).

Netrin-1 (NTN-1), a laminin-related molecule, was initially discovered as a chemoattractive or chemorepulsive cue for directing axon outgrowth and neuron migrating during the development of the nervous system (Serafini et al., 1996). NTN-1 is also known to modulate the immune response via the inhibition of inflammatory cells migration and control inflammation in non-nervous

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system diseases, such as hypoxia, acute lung injury, liver injury, peritonitis, and inflammatory bowel disease (Aherne et al., 2013, 2012; Ly et al., 2005; Mirakaj et al., 2011; Mirakaj et al., 2010; Rosenberger et al., 2009; Schlegel et al., 2016). Exogenous NTN-1 treatment has been shown to reduce the inflammation-mediated kidney injury by suppressing leukocytes infiltration and the production of inflammatory cytokine and chemokine through activating its receptor uncoordinated family member 5 B (UNC5B), which is abundantly expressed in leukocytes (Ranganathan et al., 2013a; Tadagavadi et al., 2010). A recent study reported that NTN-1 could prevent immune cells infiltration into brain parenchyma and ameliorate the severity of experimental autoimmune encephalomyelitis (EAE)-related inflammation, suggesting that NTN-1 exerts the potent anti-inflammatory property in autoimmune central nervous system diseases (Podjaski et al., 2015). Our previous study also showed that NTN-1 was upregulated in the brain and possessed neuroprotective functions of anti-apoptosis and preserving BBB integrity after SAH via distinct pathways (Xie et al., 2017b,c). Nevertheless, the effect of NTN-1 on neuroinflammation after SAH has not been determined so far.

Peroxisome proliferator-activated receptor gamma (PPARγ), belonging to the nuclear hormone receptor superfamily, is a pivotal transcription factor that regulates inflammation, apoptosis, and oxidative stress (Glatz et al., 2010; Lehrke and Lazar, 2005; Zhao et al., 2015). PPARγ was identified as a downstream molecule of NTN-1 in response to inflammation during ischemia-reperfusion (IR) injury in kidney and heart (Mao et al., 2014; Ranganathan et al., 2013b). Moreover, activation of PPARγ effectively diminished inflammation through inhibiting NFκB pathway and reducing the production of pro-inflammatory cytokines after stroke (Culman et al., 2007; Zhao et al., 2015).

In the present study, we hypothesized that exogenous recombinant human NTN-1 (rh-NTN-1) binding UNC5B receptor could attenuate neuroinflammation and early brain injury (EBI) after SAH, and the anti-inflammation mechanism of rh-NTN-1 is mediated through PPARγ/NFκB-related signaling pathway.

2. Materials and methods

2.1. Animals

All experimental protocols in this study were approved by the Institutional Animal Care and Use Committee at Loma Linda University. The study complied with the National Institutes of Health's *Guide for the Care and the Use of Laboratory Animals* and the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. Adult male Sprague Dawley rats (weight 280–330 g; Indianapolis, IN) were housed in a controlled humidity and temperature room with a 12-h light/dark cycle and *ad libitum* access to water and food.

2.2. Experimental design

Five separate experiments were performed in a rat model of SAH, as shown in Fig. 1. A total of 294 rats were used (Table 1).

Experiment 1. The role of rh-NTN-1 treatment in neuroinflammation was determined at 24 h and 72 h after SAH. Exogenous rh-NTN-1 (R&D Systems, USA) dissolved in phosphate-buffered saline (PBS) was administered through tail vein with a total volume of 200 μL at 1 h after SAH induction. Neurobehavioral function, and brain water content were examined at 24 h and 72 h post-SAH. Microglia activation was detected by immunofluorescence staining at 24 h after SAH. Rats were randomly divided into five groups: sham, SAH + PBS, SAH + rh-NTN-1 (5 μg/kg), SAH + rh-NTN-1 (15 μg/kg), and SAH + rh-NTN-1 (45 μg/kg).

Experiment 2. The time course of endogenous NTN-1, its receptor UNC5B, and PPARγ protein levels in the ipsilateral/left cerebral cortex at 3, 6, 12, 24, and 72 h after SAH were measured by Western blot analysis. The cellular localization of NTN-1 and UNC5B were detected using double immunofluorescence staining.

Experiment 3. To evaluate the effect of in vivo knockdown of endogenous NTN-1 on neuroinflammation. NTN-1 small interfering RNA (NTN-1 siRNA) was administered by intracerebroventricular injection (i.c.v.) at 48 h before SAH induction. SAH grade, neurobehavioral function, brain water content, and Western blot were measured at 24 h after SAH. Rats were randomly divided into five groups: sham, SAH + PBS, SAH + rh-NTN-1 (45 μg/kg), SAH + s crambled siRNA (Scr siRNA), and SAH + NTN-1 siRNA.

Experiment 4. To assess the role of UNC5B receptor in the neuroprotective effects of exogenous rh-NTN-1. UNC5B siRNA was administered by i.c.v. at 48 h prior to SAH induction and then followed with rh-NTN-1 (45 μg/kg) treatment at 1 h after SAH. Neurobehavioral function and brain water content were evaluated, and inflammation-related molecules were detected by Western blot at 24 h after SAH. Rats were randomly divided into five groups: sham, SAH + PBS, SAH + rh-NTN-1, SAH + rh-NTN-1 + Scr siRNA, and SAH + rh-NTN-1 + UNC5B siRNA.

Experiment 5. To explore the role of PPARγ in the anti-inflammation effect of exogenous rh-NTN-1. The selective PPARγ antagonist bisphenol A diglycidyl ether (BADGE) (30 mg/kg, Sigma-Aldrich, MO, USA) (Keep et al., 2012) dissolved in polyethyleneglycol (PEG) was injected intraperitoneally (i.p.) with a total volume of 200 μL at 1 h before SAH induction, and then followed with rh-NTN-1 (45 μg/kg) treatment at 1 h after SAH. Control animals were injected PEG (2 ml/kg) at 1 h before SAH induction and then followed with rh-NTN-1 (45 μg/kg) treatment. Neurobehavioral function, brain water content, and Western blot were evaluated at 24 h post-SAH. Rats were randomly divided into five groups: sham, SAH + PBS, SAH + rh-NTN-1, SAH + rh-NTN-1 + PEG, and SAH + rh-NTN-1 + BADGE.

2.3. SAH model

The endovascular perforation model of SAH was performed as previously described (Enkhjargal et al., 2017). Briefly, rats were anesthetized with 3% isoflurane and mechanically ventilated throughout the operation. Body temperature was maintained at 3 7 °C ± 0.5 °C using a heating lamp. A sharpened, 4–0 monofilament nylon suture was inserted into the left internal carotid artery from the external carotid artery, and then advanced 3 mm further to perforate the bifurcation of the anterior and middle cerebral arteries. In the sham group, rats were subjected to the same procedures without perforation. After the surgical procedure was completed, the rats were allowed to recover on a heated blanket.

2.4. Intracerebroventricular injection

As described previously (Huang et al., 2015), rats were anesthetized with 3% isoflurane and placed on a stereotaxic frame. The needle of a 10-μL Hamilton syringe (Microliter 701; Hamilton Company, USA) was inserted through a burr hole into the right lateral ventricle according to the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of the bregma. Following the manufacturer's instructions, a total volume of 5 μL (500 pmol) of rat NTN-1 siRNA (Thermo Fisher Scientific, USA), or UNC5B siRNA (Thermo Fisher Scientific, USA) dissolved in nuclease-free water was injected into the right ventricle by a pump at the rate of 0.5 μL/min at 48 h before SAH. The same volume of Scr siRNA (Thermo Fisher Scientific, USA) was used as a negative control. The needle was kept in place for an additional 5 min after injection to prevent possible