Brain, Behavior, and Immunity xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

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

Neuroprotective effects of nitidine against traumatic CNS injury via inhibiting microglia activation

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ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form 29 March 2015
Accepted 13 April 2015
Available online xxxx

Keywords:
Nitidine
Microglia
Traumatic brain injury
Spinal cord injury
Neuroprotection

ABSTRACT

Glial cell response to injury has been well documented in the pathogenesis after traumatic brain injury (TBI) and spinal cord injury (SCI). In the injured central nervous system (CNS), microglia and macrophages clear cellular debris and orchestrate neuronal restorative processes. However, excessive activation of these cells also increases neuroinflammatory response, expands tissue damage and hinders CNS repair. We here showed that nitidine, a benzophenanthridine alkaloid, restricted reactive microgliosis and promoted CNS repair after traumatic injury. Nitidine was shown to prevent cultured microglia from LPS-induced reactive activation by regulation of ERK and NF-κB signaling pathway. Furthermore, the nitidine-mediated inhibition of microgliosis was also shown in injured brain and spinal cord, which significantly increased neuronal survival and decreased neural tissue damage after injury. Importantly, behavioral analysis revealed that nitidine-treated mice with SCI had improved functional recovery as assessed by Basso Mouse Scale and swimming test. Together, these findings indicated that nitidine increased CNS tissue sparing and improved functional recovery by attenuating reactive microgliosis, suggestive of the potential therapeutic benefit for CNS injury.

1. Introduction

Two phases, primary and secondary injury, were involved in the pathological process of traumatic CNS injury. After a primary CNS insult, a complex series of endogenous events including reactive glial changes and neuroinflammation are triggered (Block et al., 2007; Fleming et al., 2006; Graeber and Streit, 2010; Yiu and He, 2006), leading to further neuronal degeneration and apoptosis. These subsequent pathological events, referred to as secondary injury, ultimately contribute to the failure of spontaneous anatomical and functional recovery after CNS injury. Since the initial loss of neurons caused by primary injury cannot be prevented, the main therapeutic strategy for most CNS injury is focused on alleviating deleterious effects of the secondary injury.

One of the hallmarks of secondary injury is the increased number of microglial cells and their activation at the lesion site. As the resident immunomodulatory cells in CNS, microglia plays important roles in the CNS innate immunity and response to injury (Aguzzi et al., 2013; Graeber, 2013; Graeber and Streit, 2010; Salter and Beggs, 2014). In the injured CNS, microglia and macrophages clear cellular debris and orchestrate neuronal restorative processes, while their reactive activation can also hinder CNS repair and expand tissue damage (Block et al., 2007; Hu et al., 2015; Sun et al., 2001). Microglial cells are microsensors of, and respond to, neuronal pathology (Kreutzberg, 1996). Accumulating evidence has suggested that microglial over-activation after acute CNS injury induces detrimental neurotoxic effects by releasing a diverse set of cytotoxic substances, including proinflammatory cytokine tumor necrosis factor a (TNFa), prostaglandin E2 (PGE2), and nitric oxide (NO), which lead to further neuronal loss (Aguzzi et al., 2013; Block et al., 2007; Neumann, 2001; Zhang et al., 2010). After injury, reactive microglia was also reported to produce inhibitory molecules, such as Keratan sulfate proteoglycans (KSPGs), to limit axonal regeneration (Jones and Tuszynski, 2002). Moreover, previous studies showed that release of cytokines and other soluble products by activated microglia can significantly influence the subsequent development of astrogliosis and scar formation in CNS (Rohl et al., 2007; Zhang et al., 2010; Yuan and He, 2013). The hypothesis that activated microglia initiate and maintain astrogliosis suggests that suppression of microglial over-activation might

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http://dx.doi.org/10.1016/j.bbi.2015.04.008
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effectively slow or halt the progression of reactive astrogliosis and, therefore, provide a more beneficial environment for repair after CNS injury.

As a traditional herbal medicine, *Zanthoxylum nitidium* (Roxb.) DC. has long been used for inflammatory diseases such as rheumatic arthritis and peridentitis. Nitidine is a pentacyclic alkaloid isolated from *Z. nitidium* and has been identified as a tumor-selective cytotoxic agent by potentially inhibiting the proliferative activity of tumor cells in vitro and in vivo (Chen et al., 2012; Iwasaki et al., 2010). Recently, the anti-inflammatory activity of nitidine is brought to our attention (Hu et al., 2006; Wang et al., 2012). For example, nitidine was shown to inhibit LPS-induced inflammatory cytokines production via MAPK and NF-κB pathway in RAW 264.7 cells (Wang et al., 2012). However, it is unknown whether nitidine has effects on CNS injury-induced inflammatory response, especially reactive microgliosis.

In present study, the effects of nitidine were tested on reactive microgliosis and pathogenesis in the damaged adult CNS. Our findings based on the experimental mouse model of TBI and SCI suggested that nitidine could attenuate the microglial overactivation to increase tissue sparing and improve functional recovery, shedding light on the potential therapeutic benefit for CNS trauma.

2. Materials and methods

2.1. Cell cultures

Highly enriched primary microglial cells were prepared from the cerebral cortex of postnatal day 1–3 Sprague–Dawley rats as previously described (Tamashiro et al., 2012), with minor modifications. Briefly, cerebral cortexes were dissected, digested with 0.25% trypsin, and dispersed into a single cell level. The cell suspension was filtered through a 100 μm pore mesh, then cultured with DMEM/F12 containing 10% FBS, 100U/mL penicillin and 100 μg/mL streptomycin in 75 cm^2 tissue flasks. When cells grew to confluence (7–10 d), flasks were shaken (200 rpm at 37 °C) for 6 h. The supernatant was collected and plated in culture plates for 1 h followed by lightly shaken. After discarding the less adherent cells, the attached enriched microglia were allowed to recover for 24 h and then subjected to different treatments. The purity of primary microglia is greater than 97% as determined by immunocytochemistry with antibody against Iba1. In addition, the N9 cell, a microglial cell line, was maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. To induce reactive microgliosis, primary microglia or N9 cells were stimulated with 100 ng/mL LPS (Sigma) for 24 h. To assess effects of nitidine on microglial activation, primary microglia or N9 cells were treated with nitidine (purity = 99.27%; Shanghai Winherb Medical Science Co., Ltd) at various concentration (from 0.05 to 20 μM) for indicated time course.

2.2. Immunofluorescence

For immunocytochemistry, cells cultured on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For immunohistochemistry, mice were killed by CO_2 overdose and fixed by intracardial perfusion with 4% paraformaldehyde in PBS. Brain and spinal cords were surgically dissected, post-fixed overnight and cryoprotected with 30% sucrose at 4 °C for 48 h. Tissue sections were prepared on a cryostat set at 10 μm thickness. Fixed cells or tissue sections were permeabilized and blocked with 0.2% Triton X-100 and 3% BSA in 1XPBS for 1 h, followed by overnight incubations at 4 °C with the primary antibodies listed in Supplementary Table 1. Alexa Fluor 488- or 594-conjugated corresponding secondary antibodies from Jackson ImmunoResearch were used for indirect fluorescence. For visualization of F-actin, cells were incubated with 0.1 μg/mL Rhodamine-conjugated phalloidin (Sigma) for 40 min at RT. Nuclei were counterstained with Hoechst 33342 (Hst). Images were acquired with a Nikon E600FN microscope or a Leica confocal microscopy and were analyzed using Image-Pro Plus software.

2.3. Western blot analysis

After different treatments, primary microglia cells were harvested and lysed with cold SDS gel sample buffer. The protein samples were electrophoresed on a 10% SDS PAGE gel, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and then were incubated with the indicated primary antibodies (Supplementary Table 1) overnight at 4 °C. After incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma; 1:10,000), immunoreactive bands were visualized by chemiluminescence reagents (ECL, Amersham). Immunoblot results were analyzed using Image-Pro Plus 6.0 software.

2.4. Boyden chamber migration assay

To detect the effect of nitidine on microglial motility, migration assay was performed using a 24-well Boyden chamber (Costar) containing polycarbonate membranes (8 μm pore size) as previously described (Su et al., 2007). Briefly, the polyethylene terephthalate filter membranes were coated with poly-L-lysine overnight and washed with PBS before use. Microglial cells were seeded onto the upper chamber at a density of 4×10^5 cells in 250 μL culture medium. The upper chambers were inserted into the wells which 750 μL culture medium containing 10 μM cytosine arabinoside to inhibit cellular proliferation, 100 ng/mL LPS and/or 0.1 μM nitidine was added. After incubation for 16 h at 37 °C, cells on the upper surface of membranes were removed with a cotton swab, while cells migrating through the pores to the opposite surface were fixed with 4% paraformaldehyde and stained with Coomassie Brilliant Blue. For quantitative assessment, the number of stained cells was then counted under a microscope.

2.5. Real-time PCR

Total RNA was extracted from microglia with Trizol reagent (Invitrogen) and contaminating DNA was depleted with RNase-free DNase (Fermentas). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instruction. PCR was performed to check the indicated cytokines or chemokines mRNA level using a MyiQ™ (Bio-Rad) with SYBR Green Realtime PCR Master Mix (TOYOBO Biotech) as previously described (Su et al., 2013). The assays were initiated with 1 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 15 s at 60 °C, 45 s at 72 °C. Amplification of the target cDNA was normalized to β-actin expression. Relative levels of target mRNA expression were calculated using the $2^{-\Delta\Delta C_T}$ method. For every set of PCR analysis, at least three independent experiments were performed. The primers used in this study were listed in Supplementary Table 2.

2.6. Lactate dehydrogenase (LDH) release assay

Primary microglial cells were treated with indicated concentrations of nitidine with or without 100 ng/mL LPS in culture medium for 24 h (Supplementary Fig. 3). The supernatant was collected and LDH release was detected using a CytoTox 96 Non-Radioactive

Please cite this article in press as: Yuan, Y., et al. Neuroprotective effects of nitidine against traumatic CNS injury via inhibiting microglia activation. Brain Behav. Immun. (2015), http://dx.doi.org/10.1016/j.bbi.2015.04.008