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Ginkgolide B ameliorates NLRP3 inflammasome activation after hypoxic-ischemic brain injury in the neonatal male rat

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ABSTRACT

Introduction: Perinatal hypoxic-ischemic (HI) insult is an important cause of brain injury in neonates. The development of novel treatment strategies for neonates with HI brain injury is urgently needed. Ginkgolide B (GB) is a main component of Ginkgo biloba extracts with a long history of use in traditional Chinese medicine. However, it is unknown whether GB could play a protective role in hypoxic stress in immature animals.
Methods: Using neonatal hypoxic-ischemic (HI) brain injury model of rat pups, neurological score, infarct size, and brain edema were evaluated after HI injury. The activation of microglia and the production of IL-1β and IL-18 were detected by immunohistochemistry and ELISA, respectively. A priming signal (NF-κB P65) and an activation signal (Caspase-1) of NLRP3 inflammasome activation were detected by western blot analyses.
Results: GB administrated 30 min prior to ischemia induction can improve neurological disorder, reduce infarct volume and alleviate cerebral edema. Compared with the HI groups, GB inhibited the activation of microglia and decreased the production of IL-1β and IL-18 in neocortex. Furthermore, GB reduced NLRP3 expression mainly in microglia, and significantly inhibited the expression of Caspase-1 and the nuclear translocation of NF-κB P65, preventing NLRP3 inflammasome activation.
Conclusions: GB ameliorates hypoxic-ischemic brain injury in the neonatal male rat *via* inhibiting NLRP3 inflammasome activation.

1. Introduction

Neonatal hypoxic-ischemic encephalopathy(HIE), induced by hypoxic-ischemic (HI) brain injury, is a major contributor to neonatal morbidity and mortality (Millar et al., 2017; Yildiz et al., 2017). Approximately 25% of survivors suffer from lifelong neurological deficits including neurodevelopment delays, epilepsy, cognitive issues, and motor skill (Groenendaal and de Vries, 2017). The only recognized beneficial treatment for HIE is hypothermia therapy but many infants still develop significant adverse outcomes (Galinsky et al., 2017; Manley et al., 2017). Therefore, novel therapeutic approaches directed at the pathophysiological mechanisms involved in HIE are urgently needed.

Hypoxia ischemia induces an inflammatory response in the brain and peripheral organs, resulting in the release of a number of pro- and anti-inflammatory cytokines and chemokines, including cytokines interleukin (IL)-1β and IL-18 (Ziemka-Nalecz et al., 2017; Li et al., 2017). In particular, the secretion of IL-1β and IL-18 requires the NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 3 (NLRP3) inflammasome (Singh and Jha, 2018; Song et al., 2017). NLRP3 activation has been reported to mediate injury in various CNS disorders including HIE (Ystgaard et al., 2015; Chen et al., 2018). The up-regulation of NLRP3 was found in several regions of the brain at 24 h after neonatal HI (Ystgaard et al., 2015; Chen et al., 2018). Thus, the pharmacological targeting of NLRP3-mediated inflammatory response may help design a new approach to develop therapeutic strategies for the treatment of HIE.

Ginkgo Biloba extracts (GBE) have been widely sold as phytomedicine in China and Europe (Perry and Howes, 2011; Chan et al., 2007). They have been postulated to have various effectiveness, including reduced inflammation, increased blood circulation, improvement in memory and so on (Chan et al., 2007). The most unique components of GBE are the terpene trilactones, that is, ginkgolides and bilobalide (BB) (Chan et al., 2007). As the strongest antagonist of the platelet activating

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factor, ginkgolide B (GB) possesses anti-inflammatory, anti-apoptotic, and anti-oxidant properties (Nabavi et al., 2015; Maclennan et al., 2002), but it is unknown whether GB may play a protective role in hypoxic stress in immature animals. In this study, we report that GB attenuated brain injury in the HI model, suggesting that the inhibition of NLRP3 inflammasome activation plays a crucial role in neuroprotection in the neonatal stage.

2. Materials and methods

2.1. Animals

Pregnant Sprague-Dawley (SD) rats were purchased from the Experimental Center of Yangzhou University (Jiangsu, China). The day of birth was defined as postnatal day 0 (P0). The pups were maintained at an ambient temperature of 23 ± 1 °C and a 12-hour light/dark cycle with food and water provided *ad libitum*. Because of sex-specific differences in the neonatal HI model (Hill and Fitch, 2012), only males were used in the current study. On P7, male pups were used for experiments. As a general indicator of health, body weight of pups was calculated at P7, and any pups below 4 g were not used. All experimental procedures were approved by Institutional Animal Care and Use Committee of Yangzhou University.

Experiment 1: All pups were randomly assigned to the following experimental treatment groups: a sham-operated group (vehicle, n = 8), a HI group (pups suffered from HI and vehicle, n = 16), HI + GB groups (pups suffered from HI, pre-treated with 1, 5, or 10 mg/kg GB, n = 16 per group). All pups were used to evaluate the neurological deficit, infarcted regions, and brain edema 72 h after HI injury.

Experiment 2: All pups were randomly assigned to the following experimental treatment groups: a sham-operated group (vehicle, n = 20), a HI group (pups suffered from HI and vehicle, n = 20), HI + GB groups (pups suffered from HI, pre-treated with 1, 5, or 10 mg/kg GB, n = 20 for 10 mg/kg GB, n = 16 for other groups). All pups were used for immunohistochemistry, ELISA, and western blotting 24 h after HI injury.

2.2. Drug administration

Pups were randomly selected to receive GB (purity > 99%, supplied by National Institutes for Food and Drug Control of China) or vehicle treatment. GB (1, 5, and 10 mg/kg) or vehicle control containing 5% DMSO (Dimethyl sulfoxide, D2650, purchased from Sigma Aldrich, St. Louis, MO, USA) in 0.9% saline was administered to the pups 30 min (min) prior to ischemia induction. These compounds were administered intraperitoneally (*i.p.*) with a volume to body weight injection ratio of 20 μL/g. Sham-operated animals were injected with vehicle.

2.3. Neonatal hypoxic-ischemic brain injury model

As previously described (Chen et al., 2018), hypoxic-ischemic brain injury was induced in P7 pups. Briefly, pups were anesthetized with 3% isoflurane and maintained with at 2.5% isoflurane in air during surgery. Using 5–0 surgical suture, the right common carotid artery was double ligated and transected between the ligatures. In the recovery period, pups were placed in a recovery cage to regain consciousness for 1 h. After the surgery, pups were kept in a hypoxia chamber (with 8% O_2/92% N_2) kept in a water bath maintained at 37 °C for 2.5 h. Sham animals were anesthetized and exposed, but no ligation and no hypoxia.

2.4. Neurobehavioral evaluation

Neurological score was used to evaluate the neurological deficit 72 h after HI injury. The pup was scored as 0 (normal motor function), 1 (flexion of torso and of contralateral forelimb upon lifting of the animal by the tail), 2 (circling to the ipsilateral side, but normal posture at rest), 3 (circling to the ipsilateral side), 4 (rolling to the ipsilateral side), and 5 (no spontaneous motor activity).

2.5. TTC staining, infarct size assessment and brain edema evaluation

Seventy-two hours after HI injury, whole brains were removed from eight pups per group, and were sliced into coronal sections in 1 mm. Then, sections were incubated in 1% TTC (dissolved in saline) at 37 °C in the dark for 10 min. Areas of both hemispheres and the infarcted regions were quantified for each slice using ImageJ software (NIH, MD, USA).

Seventy-two hours after HI injury, whole brains were removed from another eight pups per group. Brain edema was measured using the wet/dry weight method with water content expressed as the percent of tissue wet weight.

2.6. Immunohistochemistry

Twenty-four hours after HI injury, another eight pups per group were anaesthetized with pentobarbital sodium (80 mg/kg), transcardially perfused with saline and then 4% paraformaldehyde. After fixed in 4% paraformaldehyde at 4 °C overnight, all the brains were kept in 30% sucrose 3 days for dehydration, and sliced into coronal sections in 30 μm thickness by freezing microtome. For immunohistochemistry, the brain sections were incubated with primary antibody against Iba-1 (1:1000, Wako) at 4 °C overnight. Corresponding secondary antibody were incubated for 1 h at room temperature, and DAB staining was used to mark positive cells. The total number of Iba-1$^+$ cells was calculated with optical dissector following fractionators and a semi-automated system (Stereo Investigator, MicroBright Field. Inc., Williston, VT, USA).

For immunofluorescence, the brain sections were incubated with primary antibody against Iba-1 (1:1000, Wako) or GFAP (1:1000, Sigma-Aldrich) and NLRP3 (1:1000, AdipoGen) at 4 °C overnight, and then incubated with Alexa Fluor secondary antibodies.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Twenty-four hours after HI injury, the samples for ELISA were performed from the ischemic penumbra of eight pups per group. The samples for ELISA were performed from the ischemic penumbra of eight pups per group. Briefly, samples obtained from the border zone approximating the ischemic penumbra and the infarct core of pups were homogenized in lysis buffe (18 μl/mg tissue) containing 137 mM NaCl, 20 mM Tris − HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, leupeptin (1 μg/ml), sodium vanadate (0.5 mM), AEBSF (100 mg/ml). The homogenates were centrifuged at 12,000 × g for 10 min at 4 °C. Protein concentration was quantified with the BCA protein assay kit (#23225, Pierce Chemicals, Rockford, IL). Following the manufactures' instructions (ExCell Bio, China), the protein levels of IL-1β and IL-18 were quantitated by ELISA.

2.8. Western blotting

Twenty-four hours after HI injury, the cytoplasm and nucleus protein from the ischemic penumbra of four pups per group were collected with the nuclear and cytoplasmic protein extraction kit (Beyotime Biotechnology, China). Proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes with the electrophoretic transfer system (Bio-Rad, USA). After blocked with 10% nonfat dry milk in Tris-Hcl Buffer Saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, the membranes were then incubated with primary antibody against IL-1β (1:1000, Sigma-Aldrich, USA), Caspase-1 (1:800, Millipore, USA), NLRP3 (1:1000, AdipoGen, USA), P65 (1:800, SAB, USA), H3 (1:800, Cell Signaling Technology, Boston, USA), and β-actin (1:1000, Sigma-Aldrich, USA) overnight at 4 °C. Then, the membranes