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Research Paper

Omega-3 fatty acids regulate NLRP3 inflammasome activation and prevent behavior deficits after traumatic brain injury

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ABSTRACT

Omega-3 fatty acids (ω−3 FAs) attenuate inflammation and improve neurological outcome in response to traumatic brain injury (TBI), but the specific anti-inflammatory mechanisms remain to be elucidated. Here we found that NLRP3 inflammasome and subsequent pro-inflammatory cytokines were activated in human brains after TBI. Rats treated with ω−3 FAs had significantly less TBI-induced caspase-1 cleavage and IL-1β secretion than those with vehicle. G protein-coupled receptor 40 (GPR40) was observed to be involved in this anti-inflammation. GW1100, a GPR40 inhibitor, eliminated the anti-inflammatory effect of ω−3 FAs after TBI. β-Arrestin-2 (ARRB2), a downstream scaffold protein of GPR40, was activated to inhibit inflammation via directly binding with NLRP3 in the ω−3 FAs treatment group. Interestingly, we also observed that ω−3 FAs prevented NLRP3 mitochondrial localization, which was reversed by GW1100. Furthermore, ω−3 FAs markedly ameliorated neuronal death and behavioral deficits after TBI, while GW1100 significantly suppressed this effect. Collectively, these data indicate that the GPR40-mediated pathway is involved in the inhibitory effects of ω−3 FAs on TBI-induced inflammation and ARRB2 is activated to interact with NLRP3.

1. Introduction

Traumatic brain injury (TBI) is one of the most common life-threatening health problems in the world, and it is usually associated with a high rate of morbidity and mortality worldwide (Ghajar, 2000; Levin and Diaz-Arrastia, 2015; Lozano et al., 2015). An estimated 2 million people suffer from TBI each year in the US, and, of those, 50,000 die and 80,000 sustain permanent disabilities (Thurman et al., 1999). However, there is currently no specific treatment for patients with TBI. Standard therapy is supportive in nature and focuses on the prevention of complications (Ji et al., 2012; Lin et al., 2015). Early and effective treatment is desirable to prevent complications and facilitate good recovery. Because the primary injury is considered irrecoverable, current managements of TBI focuses on secondary injury, specifically reducing neuronal death and optimizing the neurological outcome (Ji et al., 2012; Lin et al., 2013; Lin et al., 2016). Neuroinflammation plays an important role as a mechanism of secondary injury, and it has been attributed to neurological impairment and cell death, which represents a potential therapeutic target (Esposito and Cuzzocrea, 2010; Lin et al., 2016; Lozano et al., 2015; Wang et al., 2013).

The inflammasome is a multiprotein complex. It includes a nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3), the adaptor protein ASC and caspase-1 (Wang et al., 2014; Zhou et al., 2011). Upon detecting "danger" signals, the inflammasome is assembled and activated to promote the maturation of caspase-1, which then results in the secretion of several pro-inflammatory cytokines, including interleukin-1 (IL-1) and IL-18 (Yuan et al., 2015; Zhou et al., 2011).

Omega-3 fatty acids (ω−3 FAs), including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and other family members, are essential to brain development and plasticity (Dyall and Michael-Titus, 2008; Innis, 2007). Some previous researches in preclinical animal studies or in young children with ω−3 FAs deficiencies demonstrated that long-term supplementation can significantly improve memory and learning function (Hashimoto et al., 2002; Lim et al., 2005; Stonehouse, 2014). Accumulating evidence have shown that ω−3 FAs could suppress inflammation and then improve neurological recovery (Begum et al., 2014; Lewis et al., 2013; Wu et al., 2011). However, the

Abbreviations: ω−3 FAs, omega-3 fatty acids; TBI, traumatic brain injury; CCI, controlled cortical impact; IL-1β, interleukin-1β; GPR40, G protein-coupled receptor 40; ARRB2, β-arrestin-2; NLRP3, nucleotide-binding domain and leucine-rich repeat containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD.

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specific molecular mechanism involved in the regulation of inflammation remains poorly understood.

Based on these observations and previous studies (Begum et al., 2014; Lewis et al., 2013; Yan et al., 2013), we hypothesized that ω−3 FAs could ameliorate TBI-induced neuronal death and improve outcome via repressing inflammation. To address these questions, experimental rats were randomly allocated to receive either standard diet or high ω−3 FAs diet. Small interfering RNA (siRNA) or drugs were applied to inhibit the ω−3 FAs anti-inflammatory effect.

2. Materials & methods

All experiments of patients and rats were approved by the ethics committee of Nanjing Medical University and Second Military Medical University. The experimental procedures were conducted in accordance with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). All rats had free access to water and food, and were kept at room temperature of 25 ± 2 °C and a standard 12-h light/dark cycle. After surgery, the injured rats were housed in separate cages from the uninjured ones under the same conditions. All efforts were made to minimize pain and suffering of rats in this research.

2.1. Human brain tissues

These brain samples were obtained from the department of neurosurgery, the First Affiliated Hospital of Nanjing Medical University. All experiments of human brain tissues were approved by the First Affiliated Hospital of Nanjing Medical University and performed in accordance with its guidelines. Informed consent was obtained directly from each patient or family members, and documented in writing before experiments.

2.2. Animal model

The CCI model, an in vivo TBI model, was performed as previously described (Ji et al., 2012). Eight-weeks-old male Sprague-Dawley rats weighing 220–260 g were anesthetized with 4% isoflurane in 70% N_2O and 30% O_2, and anesthesia was maintained using 2% isoflurane. A 10 mm craniotomy was made over the left parietal cortex using a dental drill. Rats were subjected to CCI with a 6-mm metal impounder, 6.0 ± 0.2 m/s velocity, 2.5 mm depth, and 50 ms duration. After surgery, the bone flap was replaced and rats returned to their cages. There was a sham group for each experiment. All rats except for behavioral tests were sacrificed at 8 h post-injury.

2.3. Animal treatment

On Day 1 of pregnancy, mothers were randomly allocated to receive either standard (EPA 20:5 n−3 < 0.02% and DHA 22:6 n−3 < 0.02% of total free fatty acids), or high ω−3 FAs diet (EPA 5.3% and DHA 23.8% of total free fatty acids) (Jones et al., 2013).

The standard (AIN93G), high ω−3 (SF12-037), and high ω−6 (SF14-162) diets were obtained from specialty feeds, Glen Forrest, Australia. Both high ω−3 and high ω−6 diets formulations were based on AIN-93G. The entire contents, vitamins, dietary fats, and lipid profile of different diets were listed in Supplemental Tables 1, 2, 3, and 4. The ω−3 (DHA, 22:6, #D2534), ω−6 (oleic acid, 18:2, #62160), and ω−9 (linolenic acid, 18:1, #O1008) for the gavages (100 mg/kg, twice a week for 6 weeks before surgery) were supplied by sigma (ALDRICH, USA). In this study, rats of sham group were fed with standard diet (AIN93G). PBS was used for the gavages. These rats underwent anesthesia and "sham-surgery" under the same condition. The rats of high ω−9 group were fed with AIN93G and followed by ω−9 administration via gavages for 6 weeks before surgery. Experimental rats were also fed with a standard or high FAs diets after surgery.

GW1100 (#10008908), a selective GPR40 antagonist, was obtained from Cayman Chemical Co. and dissolved in dimethyl sulfoxide. It (10 μg/rat) was administered via the intracerebroventricular route 30 min before brain injury. After injury, experimental rats for behaviors tests were continually given GW1100 (20 μg/rat) or dimethyl sulfoxide via intraperitoneal injection twice a day.

2.4. siRNA administration into rat lateral ventricle

Osmotic mini pumps (Alzet model 1003D) were obtained from Durect, Cupertino, CA. The pumps were filled with the 30 nmol siRNA for infusion and implanted into the left lateral ventricle at a rate of 1.0 μL/day for 72 h before injury in accordance with the manufacturer instructions. Scrambled siRNA was applied as control. The targeted protein was assessed by western blot.

2.5. Cytokines in cerebrospinal fluid

Cerebrospinal fluid (CSF) was withdrawn from the cisterna magna under anesthesia and immediately flash frozen in liquid nitrogen. The levels of IL-1β (R&D Systems), IL-6 (R&D Systems) and IL-18 (Thermo Fisher scientific) in CSF were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Briefly, samples were incubated at room temperature for 2 h. The absorbance was detected at 450 nm within 15 min after adding the stop solution.

2.6. Western blot

Thirty micrograms of proteins for each sample, including both human brains and rat cortex, were separated using a 10% or 12% SDS-PAGE gel (BIO-RAD) and then transferred onto 0.45 μm polyvinylidene fluoride membranes. After being incubated with primary antibodies overnight at 4 °C, the membranes were probed by secondary antibody at room temperature for 1 h. The band densities were evaluated with Image J (National Institutes of Health, Bethesda, MD, USA).

The antibodies used were as follows: GAPDH (Cell Signaling Technology, #5174, 1:2000), IL-1β (1:1000, ab200478, Abcam), IL-1β (1:1000, NBP2-27342, Novus Biologicals), caspase-1 (1:1000, #2225, Cell Signaling Technology), caspase-1 (1:1000, NBP1-45433, Novus Biologicals), NLRP3 (1:1000, NBP2-12446, Novus Biologicals), ARRB2 (1:1000, #4674, Cell Signaling Technology), and GPR40 (1:1000, NBP1-00860, Novus Biologicals), caspase-3 (1:1000, #9662, Cell Signaling Technology).

2.7. TUNEL staining and immunohistochemistry

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to assess apoptotic cell death according to the manufacturer's instruction (#12156792910, Roche, Germany). The rats were sacrificed and perfused with 0.1 mM PBS (PH 7.4). In brief, coronal frozen sections (7 μm thick) were embedded onto the slides and incubated with 50 μL TUNEL reaction mixture. The slides were observed and quantified using a Nikon fluorescent microscope. The apoptotic cells showed red fluorescence. The nuclei were stained with DAPI and showed blue fluorescence. The apoptosis was calculated as TUNEL positive cells/DAPI.

Immunohistochemistry was performed to evaluate TBI-induced inflammation. Human brain tissue sections were incubated by the following primary antibodies: anti-NLRP3 (1:200, NBP2-12446, Novus Biologicals), anti-IL-1β (1:200, NBP1-19775, Novus Biologicals) and IL-18 (1:200, NBP2-38481, Novus Biologicals). After primary antibodies, the sections were washed with PBS, and then incubated with secondary antibody. The number of positive cells was counted from five randomly chosen fields, and the average number was calculated.