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NLRP3 INFLAMMASOME ACTIVATION MEDIATES FATIGUE-LIKE BEHAVIORS IN MICE VIA NEUROINFLAMMATION

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Abstract—Numerous experimental and clinical studies have suggested that the interaction between the immune system and the brain plays an important role in the pathophysiology of chronic fatigue syndrome (CFS). The NLRP3 inflammasome is an important part of the innate immune system. This complex regulates proinflammatory cytokine interleukin-1β (IL-1β) maturation, which triggers different kinds of immune-inflammatory reactions. We employed repeated forced swims to establish a model of CFS in mice. NLRP3 knockout (KO) mice were also used to explore NLRP3 inflammasome activation in the mechanisms of CFS, using the same treatment. After completing repeated swim tests, the mice displayed fatigue-like behaviors, including locomotor activity and reduced fall-off time on the rota-rod test, which was accompanied by significantly higher mature IL-1β level in the prefrontal cortex (PFC) and malondialdehyde (MDA) level in serum. We also found increased NLRP3 protein expression, NLRP3 inflammasome formation and increased mature IL-1β production in the PFC, relative to untreated mice. The NLRP3 KO mice displayed significantly moderated fatigue behaviors along with decreased PFC and serum IL-1β levels under the same treatment. These findings demonstrated the involvement of NLRP3 inflammasome activation in the mechanism of swimming-induced fatigue. Future therapies targeting the NLRP3/IL-1β pathway may have significant potential for fatigue prevention and treatment.

Key words: NLRP3 inflammasome, fatigue, IL-1β.

INTRODUCTION

Fatigue is a widespread disorder and a major contributor to the global burden of disease. Fatigue symptoms are related to central nervous system (CNS) dysfunction, including reduced activities and muscle endurance (Afari and Buchwald, 2003). In addition to fatigue, a spectrum of fatigue-based syndromes including depression, sleep disturbances, malaise and gastrointestinal symptoms are known to contribute to chronic fatigue syndrome (CFS), which affects 1% of the US population. Women are four times as likely as men to suffer from fatigue (Cleare et al., 2015).

Although randomized controlled trials aiming at positive therapeutic outcomes through antiviral therapy, immunological therapy, exercise therapy and antioxidant therapy have achieved significant progress, the results in treating fatigue are not yet satisfactory (Henderson, 2014; Lloyd and Meer, 2015; Castro-Marrero et al., 2016; Larun et al., 2016; Mitchell, 2016). Therefore, there remains a need for a more comprehensive understanding of fatigue mechanisms.

Research over the past decade has confirmed that oxidative stress and inflammation are indispensable elements in fatigue (Morris et al., 2016). Elevated oxidative stress levels could result in the activation of immune-inflammatory pathways and neurotransmitter dysfunction, which induce fatigue behaviors. Several lines of evidence have illustrated increased expression of pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in the serum of CFS patients (Ousman and Kubes, 2012). Further evidence from animal experiments indicated that increased levels of IL-1β were the key stimulus in a poly I:C-induced rodent fatigue model (Ifuku et al., 2014; Vasiadi et al., 2014). Moreover, many studies have reported that high-intensity exercise could result in pro-inflammatory cytokine production and increased oxidative stress, resulting in aggravated feelings of fatigue (Nieman et al., 2005; Spence et al., 2007). Based on these findings, ibuprofen has been widely used during long-term intensive exercise (Lima et al., 2016). All these lines of evidence have provided a comprehensive understanding of the pathophysiologic mechanisms of fatigue, indicating that immune-inflammatory disorder plays a key role in the pathogenesis of fatigue.

The NLRP3 inflammasome is an integral part of the innate immune system. The NLRP3 inflammasome is assembled by the pattern recognition NLRP3 receptor, the adaptor protein ASC, and the effector protein

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Abbreviations: CFS, chronic fatigue syndrome; IL-1β, interleukin-1β; KO, knockout; LPS, lipopolysaccharide; MDA, malondialdehyde; PFC, prefrontal cortex; ROS, reactive oxygen species.

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pro-caspase-1, which triggers the maturing and release of IL-1β and IL-18 (Coll et al., 2016). It could be activated by a series of invasive pathogens, as well as endogenous damage signals including mitochondrial dysfunction and the production of reactive oxygen species (ROS). Both of these have been reported as important contributors in the development of fatigue (Morris and Maes, 2014). Studies have demonstrated that NLRP3 inflammasome signaling contributes to autoimmune and neurodegenerative diseases such as gout, type II diabetes and Alzheimer's disease (Olsen and Singhrao, 2016). The NLRP3 inflammasome also has been implicated as a promising target for disease treatment.

Our previous work proved that NLRP3 inflammasome activation was involved in a lipopolysaccharide (LPS)-induced mouse fatigue model (Zhang et al., 2016). We assumed that the NLRP3 inflammasome might also be a key component in exercise-induced fatigue. Therefore, the present study was designed to detect what role NLRP3 inflammasome might play in fatigue induced by repeated forced swims, with the help of NLRP3 knockout (KO) mice.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old female C57BL/6 mice (wild-type, hereafter WT) purchased from Shanghai Super-B&K Laboratory Animal Corp., Ltd. were housed in a specific-pathogen-free animal facility with 12-h light–dark cycles (light from 8:00 a.m. to 8:00 p.m.). Food and water were provided *ad libitum*, unless otherwise described. NLRP3 KO mice of similar age and weight purchased from the Model Animal Research Center of Nanjing University (AAALAC accredited) were also housed in the same animal facility.

Experimental groups

The animals were randomly divided into four groups: WT control (WT/no swim), NLRP3 KO control (*NLRP3*$^{-/-}$/no swim), WT with repeated forced swim (WT/swim) and NLRP3 KO with repeated forced swim (*NLRP3*$^{-/-}$/swim). There were 6–8 animals in each group. Before the rota-rod test, the mice were subjected to adaptive training. The training lasted for 5 min at a speed of 10 rpm. The experiment protocol was approved by the Animal Care Committee of Second Military Medical University and all procedures were carried out in accordance with related regulations and guidelines.

Reagents

In western blot analysis, the primary antibodies were anti-NLRP3 purchased from AdipoGen Corp, (San Diego, CA, USA); anti-IL-1β purchased from Cell Signalling Technology (Beverly, MA, USA) and anti-actin from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). For immunofluorescence staining, anti-NLRP3 was the same in western blot analysis. The anti-ASC (sc-22514) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Alexa Fluor 488 anti-rabbit, Alexa Fluor 555 anti-mouse and Alexa Fluor 647 anti-rabbit were obtained from Life Technology (Shanghai, China). The serum malondialdehyde (MDA) measurement kit came from Xi Tang Biotechnologies Co., Ltd. (Shanghai, China). All the reagents listed above were used according to the manufacturers' instructions.

Repeated forced swim treatment

Repeated forced swim treatment was performed to induce fatigue according to the methodology described in previous studies, with several modifications (Li et al., 2015; Sarvaiya and Goswami, 2016). The forced swim treatment was carried out for 10 min every 12 h for 14 consecutive days. The mice were forced to swim in a glass rectangular jar (25 × 31 cm) containing water 25 cm deep. The water temperature was controlled at 25 ± 2 °C. To prevent the mice from floating, a light lead sinker (6–10% of the mouse's weight) was tied to the tail root of each mouse during the swim treatment. The water was deep enough to keep the animals from touching the bottom of the jar with their tails. The mice were wiped with a clean towel after the forced swim treatment.

Locomotor activity test

The locomotor activity was conducted for 30 min once a day during the dark portion of the day, using the Neuroscience Behaviour Mice Cage Rack System (ShangHai Biowill Co., Ltd., Shanghai, China). The traveling video was then recorded and analyzed by Motor Monitor software (Shanghai Biowill Co., Ltd.) The distance that each mouse traveled could also be spontaneously calculated. The average distance over 14 consecutive days was used for data analysis. The locomotor activity was carried out for 30 min once a day during the dark cycle. The average distance covered in 30 min was calculated and used in the statistical analysis.

Rota-rod test

The rota-rod test was performed once a day, after the forced swim treatment. The rod's speed was set at 30 rpm. The elapsed time before each mouse fell off the rod was recorded. The longest time period for which any animal could remain on the rod was 600 s. The average fall-off time over 14 consecutive days was used for data analysis.

Sample collection

At the end of the two weeks of forced swim treatment, the mice were sacrificed under general anesthesia with pentobarbital sodium (100 mg/kg). For ROS detection, the brain was immediately extracted from each animal, flash-frozen in liquid nitrogen, and then stored. For immunofluorescence staining, the perfusion was performed with a saline solution (0.9%) via the mouse's heart and the brain was then fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) at room temperature. Blood was collected by cardiac puncture and was then