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Manganese exposure facilitates microglial JAK2-STAT3 signaling and consequent secretion of TNF-a and IL-1β to promote neuronal death

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ABSTRACT

Chronic manganese (Mn) exposure can lead to neuroinflammation and neurological deficit, which resemble idiopathic Parkinson's disease (IPD). However, the precise mechanisms underlying Mn exposure-induced neurotoxicity remain incompletely understood. Microglia can become hyperactivated and plays a vital role in neuroinflammation and consequent neurodegeneration in response to pro-inflammatory stimuli. In the present study, we found that HAPI microglial cells exhibited increased secretion of pro-inflammatory TNF-α and IL-1β following Mn exposure in dose- and time-dependent manners. In addition, we showed that Mn exposure could trigger the activation of JAK2/STAT3 signaling pathway in microglia. Notably, Mn-induced secretion of TNF-α and IL-1β was significantly attenuated by the treatment of JAK2 inhibitor. Finally, through incubating PC12 neuronal cells with Mn-treated microglial conditioned medium, we demonstrated that Mn-induced secretion of microglial TNF-α and IL-1β facilitated neuronal apoptosis. Thus, we speculate that Mn exposure might trigger JAK2-STAT3 signal pathway in microglia, leading to resultant neuroinflammation and neuronal loss.

1. Introduction

Manganese (Mn) is an essential trace metal that plays an important role in mediating many physiological functions. However, accumulation of excess Mn in the brain may lead to neurological disorders known as manganism, which have some similar features of idiopathic Parkinson's disease (IPD) (Bouabid et al., 2015). Short periods of low-dose Mn exposure may not necessarily cause subclinical Parkinsonism. However, brain is sensitive to chronic Mn overload. Sustained Mn exposure beyond the physiological needs will lead to neurotoxicity (Greiffenstein and Lees-Haley, 2007; Ma et al., 2015). Many epidemiological studies on chronic manganese exposure concerned its effect on occupational workers. Miners and welders who are continuously exposed to Mn are at high risk of Mn-induced neurotoxity (Greiffenstein and Lees-Haley, 2007; Meyer-Baron et al., 2009). Additionally, other occupational manganese exposure also causes important public health concern. However, the exact mechanism underlying Mn-induced neurotoxity remains to be elucidated. Many investigations have indicated that apoptotic neuronal death plays an important role in pathophysiological process of Mn neurotoxicity (Zhao et al., 2009). However, little is known regarding the effect of chronic manganese exposure on microglial cells. Studies have reported that neuroinflammation contribute to progressive neurodegeneration in several neurodegenerative disorders (McGeer and McGeer, 2008).

Microglia are resident innate immune cells in the central nervous system (CNS). Microglial activation plays a vital role in neuroinflammation and consequent neurodegeneration by producing cytokines and chemokines (Hines et al., 2009), leading the activation of astrocytes and the recruitment of peripheral immune cells. The activation of microglia is initiated by various pathological factors (Perry and Holmes, 2014). Under the normal physiological condition, microglia exist in a quiescent state and monitor the surrounding environment. However, microglia multiply and adopt an activated state in response to unfavorable stress conditions or neurotoxin exposure (Yang et al., 2010). Microglia undergo several changes, including morphological changes and the expression of surface antigens, which were considered as the hallmarks of

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microglial activation. Microglia can display diverse functional phenotypes that range from neuroprotective M2 phenotype to pro-inflammatory M1 phenotype. M1 microglia can produce many pro-inflammatory cytokines, such as TNF-α, IL-1β, NO and ROS, contributing to chronic inflammatory environment (Garden and Moller, 2006; Tang and Le, 2016; von Bernhardi et al., 2015). This classical phenotype of microglia plays an important role in the pathogenesis of various neurodegenerative diseases, such as Parkinson's disease (Tansey et al., 2007), Alzheimer's disease (Mhatre et al., 2004), and amyotrophic lateral sclerosis(McGeer and McGeer, 2002).

The mechanisms underlying microglial activation and consequent cytokine release following Mn exposure remain largely unclear. In response to extracellular stimuli, several studies have shown that many major signaling pathways are initiated in activated microglia, such as NF-kB and AP-1 (Chang et al., 2008) (Lee et al., 2005) (Yang et al., 2010). The JAK/STAT signaling pathway is another critical pro-inflammatory signaling pathway, orchestrating adaptive immune mechanisms. Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) are important pro-inflammatory signaling molecules and mediate the expression of approximately 60 cytokines (O'Shea and Plenge, 2012). STAT proteins, predominantly STAT3, are latent transcription factors in the cytoplasm that become activated after tyrosine phosphorylation by members of JAK family, and then lead to transcriptional activation (Heinrich et al., 2003; Levy and Darnell, 2002). What's more, the upregulation of STAT3 has been detected in neuropathological conditions including Parkinson's disease (Qin et al., 2016), Hypoxia-ischemia (HI) (D'Angelo et al., 2016) and Septic shock (Beurel and Jope, 2009). Although the contribution of JAK/STAT signaling pathway on inflammation has been widely acknowledged, it remain unclear whether JAK/STAT3 signaling pathway is involved in neuroinflammation following chronic Mn exposure.

We undertook studies to investigate the molecular mechanisms underlying Mn-induced neuroinflammation. Herein, we investigated the detailed contribution of JAK/STAT3 signaling pathway on Mn-induced neuroinflammation. We showed that STAT3 was activated *in vivo* and *in vitro* following chronic manganese exposure. Our study may provide a detail insight into the mechanisms underlying Mn neurotoxicity.

2. Materials and methods

2.1. Animal model

Twenty-four male Sprague-Dawley (SD) rats (4/group, 4-week old, weighting approximate 200–230 g) obtained from Medical College of Nantong University were used in all experiments. Rats were acclimated for 1 week to the air-conditioned room (22 ± 1 °C) on a 12 h light/dark cycle with water and food available freely. The rats were randomly divided into four groups: control group and Mn-exposed groups. For the control group, each rats were received 0.9% normal saline, while Mn-exposed groups received 2, 5 and 10 mg/kg $MnCl_2$ via intragastric gavage daily for 30 days. Finally all rats were deeply anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg, i.p.; Lundbeck) and sacrificed to obtain the brain tissues. All procedures involving animal studies was executed according to the guidelines of National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and therefore approved by the Animal Care and Use Committee of Nantong University, China.

2.2. Cell culture and experimental treatments

The HAPI cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Shanghai, China) supplemented with10% (v/v) fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) in a 5% CO_2 humidified incubator at 37 °C. Then the cells were treated with varying concentrations of $MnCl_2$ (50, 100, 300, 500, and 1000 μM) for 12 h or different times (2, 4, 6, 9, 12 and 24 h) with 500 μM and both control group was treated with fresh medium. For JAK2 inhibitor treatment, the HAPI cells were pretreated with AG490 (50 μM, 1 h) before Mn exposure. The protein samples were stored at −80 °C for further use.

The PC12 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (GIBCO, Shanghai, China) supplemented with10% (v/v) fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) in a 5% CO_2 humidified incubator at 37 °C. Then the PC12 cells were exposure to conditioned medium of HAPI cells, which were treated with 500 μM $MnCl_2$. And some of which were preconditioned with neutralizing antibody against IL-1β and TNF-α for 1 h after treated with 500 μM $MnCl_2$ for 12 h.

2.3. Western blot analysis

Before harvest, HAPI cells were washed with ice-cold PBS and then lysed with lysis buffer containing protease inhibitors for 30 min on ice. The samples were centrifuged at 13,000 rpm, 4 °C for 15 min. Then the protein concentrations of the supernatants were determined by using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). The protein samples were separated by SDS-PAGE gels and were transferred to PVDF membranes by a transfer apparatus at 300 mA for 1.5 h. Then the membranes were blocked with 5% Skim Milk in PBST at room temperature (RT) for 2 h and were incubated with the following primary antibodies at 4 °C overnight: anti-GAPDH (1:2000; Santa Cruz), anti-β-actin (1:2000; Santa Cruz), anti-phospho-STAT3 tyr-705 (1:1000; Abcam), anti-STAT3 (1:1000; Santa Cruz), anti-JAK2 (1:1000; Santa Cruz), anti-phospho-JAK2 Tyr-1007/1008 (1:1000; Abcam), anti-cleaved PARP (1:1000; Cell Signal) and anti-active Caspase-3 (1:500; Santa Cruz). Subsequently, the membranes were incubated with the appropriate secondary antibodies for 2 h at room temperature. Followed by detection with enhanced chemiluminescence, the intensity of relative protein expression was detected by densitometry (Image J, NIH, Bethesda, MD, USA).

2.4. Immunofluorescent staining

After treatment by $MnCl_2$, HAPI cells plated on glass coverslips were fixed with 4% paraformaldehyde for 1 h at 4 °C. After washed with ice-cold PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and then blocked with 5% normal donkey serum for 2 h at room temperature. Subsequently, the cells were incubated with anti-STAT3 antibodies (1:100; Santa Cruz) at 4 °C overnight. After washing with ice-cold PBS for 3 times, the cells were incubated with TRITC-conjugated secondary antibodies (1:500 dilution in PBS) and Hoechst 33258 (Invitrogen, 1:1000) for 2 h at 25 °C. At last, stained cells were visualized by using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar,Germany).

2.5. Enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-1β

The concentrations of TNF-α and IL-1β released in cultured supernatants were determined by a rat TNF-α and IL-1β ELISA kit (ExCell Biology, Shanghai, China). Briefly, the culture supernatants (100 μL) of the treated cells were collected and evaluated by multiplex ELISA according to the manufacturer's protocol.

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