Original Article

Lead exposure induces cell autophagy via blocking the Akt/mTOR signaling in rat astrocytes

Yingying Huang^{1,2}, Yingjun Liao¹, Huijun Zhang² and Shuyun Li²

¹Department of Physiology, School of Life Sciences, China Medical University, Shenyang, Liaoning, 110122, China

²School of Nursing, Jinzhou Medical University, Jinzhou, Liaoning, 121001, China

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ABSTRACT — Lead is a main threat to human health due to its neurotoxicity and the astrocyte is known to be a common deposit site of lead *in vivo*. However, the detailed mechanisms related to lead exposure in the astrocytes were unclear. In order to deeply investigate this issue, we used Sprague-Dawley (SD) rats and astrocytes isolated from the hippocampus of SD rats to establish the lead-exposed animal and cell models through treating with lead acetate. The expression levels of GFAP, LC3, and p62 in the rat hippocampus were detected by immunofluorescence and Western blot after lead exposure. The effects of autophagy on lead-exposed astrocytes were studied by further autophagy inhibitor 3-methyladenine (3-MA) induction. Transmission electron microscopy was used to observe autophagosomes in astrocytes after lead acetate treatment, followed by assessing related autophagy protein markers. In addition, some inflammatory cytokines and oxidative stress markers were also evaluated after lead exposure and 3-MA administration. We found that lead exposure induced activation of astrocytes, as evidenced by increased GFAP levels and GFAP-positive staining cells in the rat hippocampus. Moreover, lead exposure induced autophagy in astrocytes, as evidenced by increased LC3II and Beclin 1 protein levels and decreased p62 expression in both the rat hippocampus and astrocytes, and it was confirmed that this autophagy was activated through blocking the downstream Akt/target of the rapamycin (mTOR) pathway in astrocytes. Furthermore, it was shown that treatment of lead acetate increased the release of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), and the accumulation of malondialdehyde (MDA) and myeloperoxidase (MPO) in astrocytes, which could be alleviated by further 3-MA induction. Therefore, we conclude that lead exposure can induce the autophagy of astrocytes via blocking the Akt/mTOR pathway, leading to accelerated release of inflammatory factors and oxidative stress indicators in astrocytes.

Key words: Lead exposure, Astrocytes, Autophagy, The Akt/mTOR signaling, Inflammatory cytokines, Oxidative stress

INTRODUCTION

Lead is a well-known human neurotoxin usually used in the battery, paint, ammunition, and plastic manufacturing industries (Abadin *et al.*, 2007). Due to its widespread use and hard degradation, long-term accumulation causes pollution to the atmosphere and water resources. People may be exposed to lead and lead-containing chemicals via breathing, drinking, eating, or swallowing lead-containing dust or dirt (Abadin *et al.*, 2007). Lead poisoning is the result of prolonged exposure to lead, and its primary target is the nervous system. When lead levels in adult blood exceed 60 $\mu\text{g/dL}$, various neurological diseases may be triggered (Spivey, 2007). Children have a high-

er risk of lead poisoning than adults because the developing nervous system is more sensitive to lead (Abadin *et al.*, 2007; Murata *et al.*, 2009). Glial cells are one of the main cells in the central nervous system, including astrocytes, oligodendrocytes, and microglia (Wei *et al.*, 2014). In the central nervous system, astrocytes are more likely to accumulate lead than neuronal cells (Lindahl *et al.*, 1999). Therefore, lead may damage the nervous system by acting on astrocytes.

Astrocytes not only provide nutritional support for the central nervous system, but also perform other functions, such as neurotransmission, cellular signaling, inflammation, and synaptic regulation (Ricci *et al.*, 2009). Under normal conditions, the central nervous system has a potent

Correspondence: Yingjun Liao (E-mail: yjliao@cmu.edu.cn)

anti-inflammatory effect, but under threat, astrocytes are activated and assume some of the functions of immune cells, including the release of pro-inflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) (Jensen *et al.*, 2013). The gradual activation of astrocytes and the subsequent overproduction of pro-inflammatory factors lead to neuroinflammation and further neuron dysfunction (Ahmad *et al.*, 2019; Hostenbach *et al.*, 2014). Periyasamy and colleagues indicated that cocaine-induced activation of astrocytes through endoplasmic reticulum stress-mediated autophagy activation, and further induced the release of IL-1 β , IL-6, and TNF- α (Periyasamy *et al.*, 2016). Their findings suggested that the induction of autophagy is important for activation of astrocytes and release of inflammatory cytokines.

Autophagy is a self-degrading process which has been reported to be induced in various physiological diseases, including cancer and neurodegenerative disorders (Mizushima and Komatsu, 2011; Sung and Jimenez-Sanchez, 2020). Mechanistic studies suggested that autophagy was associated with oxidative stress, inflammatory response, and astrocyte function (Glick *et al.*, 2010; Wang and Xu, 2020). Many environmental factors can induce autophagy, such as hepatitis C virus, radiation, and methylmercury (Chan and Ou, 2017; Chaurasia *et al.*, 2016; Lin *et al.*, 2019). However, whether autophagy is induced by lead exposure in astrocytes remains unclear. Early studies showed that lead exposure induced autophagy in lung cancer cells and osteoblasts (Li *et al.*, 2016; Lv *et al.*, 2015). Moreover, acute lead poisoning in the brains of adult rats induced reactive astrocytes and triggered endoplasmic reticulum stress in astrocytes (Qian and Tiffany-Castiglioni, 2003; Struzyńska *et al.*, 2001). Therefore, we hypothesized that lead exposure induced autophagy in astrocytes and further increased the release of inflammatory cytokines.

MATERIALS AND METHODS

Animal grouping and treatment

The present study was approved by the Institutional Animal Care and Use Committee of the China Medical University and carried out according to the Guideline for the Care and Use of Laboratory Animals. The 12 male Sprague-Dawley rats, weighing about 250 g, were randomly divided into two groups. The control group was given normal drinking water, while rats in the lead acetate group were given drinking water containing 300 ppm of lead acetate for 30 days (Ademuyiwa *et al.*, 2009; Fiorese *et al.*, 2014). Finally, the rats were sacrificed, and hippocampal tissue was isolated for subsequent experiments.

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Immunofluorescence staining

The hippocampal tissue was paraffin-embedded and sectioned (5 μ m). Sections of hippocampal tissue were treated with goat serum for 15 min. After washing 3 times with PBS (5 min/time), the sections were incubated with anti-microtubule-associated protein light chain 3 (LC3) primary antibody (1:200, Proteintech, Wuhan, China) and anti-glial fibrillary acidic protein (GFAP) primary antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ionized calcium-binding adapter molecule 1 (Iba-1) primary antibody (1:300, Cell Signaling Technology (CST), Danvers, MA, USA) or anti-neuronal nuclei (NeuN) primary antibody (1:300, CST) at 4°C overnight. After washing 3 times with PBS (5 min/time), the sections were incubated with secondary antibodies for 90 min at room temperature. Cy3-labeled secondary antibody (1:200, Beyotime, Shanghai, China) combined with anti-LC3 primary antibody, and FITC-labeled secondary antibody (1:200, Beyotime) combined with anti-GFAP primary antibody. The sections were washed with PBS for 3 times (5 min/time), and the nuclei were stained with DAPI (Beyotime) for 30 min. The sections were washed with PBS for 3 times (5 min/time), and then anti-fluorescence quenching agent was added to seal the slices. Finally, the staining effect was observed and photographed under a fluorescence microscope (Olympus, Tokyo, Japan) ($\times$ 400).

For p62 and GFAP staining in astrocytes, the cells were fixed with 4% paraformaldehyde for 15 min and then treated with 0.1% Triton X-100 for 30 min. After washing with PBS, the cells were incubated with goat serum for 15 min. Subsequently, cells were treated with anti-p62 primary antibody (1:200, Proteintech) or anti-GFAP primary antibody (1:200, Santa Cruz Biotechnology) overnight at 4°C, followed by Cy3-labeled secondary antibody (1:200, Beyotime). The rest of the steps are the same as above.

Western blot analysis

Total protein was extracted from hippocampal tissue and astrocytes using radioimmunoprecipitation assay lysis buffer and phenylmethanesulfonyl fluoride protease inhibitor. The protein lysate was assayed for protein concentration using the Bicinchoninic Acid Protein Concentration Kit (Solarbio, Beijing, China) according to the manufacturer's instructions. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membranes. The membranes were then incubated with primary antibody at 4°C overnight followed by secondary

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antibody. The primary antibodies used were as follows: p62 antibody (1:1000, CST, Danvers, MA, USA), Beclin 1 antibody (1:1000, CST), LC3 antibody (1:1000, CST), Akt (1:2000, CST)/p-Akt antibody (1:1000, CST), target of rapamycin (mTOR) (1:1000, CST)/p-mTOR antibody (1:500, CST), p70S6K (1:1000, CST)/p-p70S6K antibody (1:500, CST), and GAPDH (1:10000, Proteintech). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit/mouse secondary antibodies (1:3000, Solarbio). Finally, the proteins on the membranes were visualized with enhanced chemiluminescence detection (ECL) reagents, and the optical density values of the target bands were analyzed by gel-pro-analyzer software.

Astrocytes isolation and treatment

The isolation of astrocytes was performed as previously described (Chen *et al.*, 2018; Wang *et al.*, 2020). In brief, the astrocytes were isolated from the hippocampus of Sprague-Dawley rats one day after birth. The astrocytes were passed through 3 times for further purification so that the culture containing 98% > astrocytes was obtained and verified by GFAP immunofluorescence staining. The isolated astrocytes were cultured in a Dulbecco's Modified Eagle's medium medium containing 10% fetal bovine serum and then placed in an incubator at 37°C, supplied with 5% CO₂. The astrocytes were treated with lead acetate (1 µM or 10 µM) for 24 hr or treated with lead acetate (10 µM) and autophagy inhibitor 3-methyladenine (3-MA) (5 mM) simultaneously for 24 hr (Hossain *et al.*, 2000; Posser *et al.*, 2007).

Transmission electron microscopy detection

The formation of autophagosomes in astrocytes was observed with a transmission electron microscope. Briefly, the astrocytes were fixed in 2.5% glutaraldehyde for 5 min at 37°C. The cells were then dehydrated and embedded in resin and cut into ultrathin sections. The sections were stained with lead citrate and examined under a transmission electron microscope (Hitachi, Tokyo, Japan).

ELISA assay

The expression levels of IL-1β and TNF-α in rat astrocytes were measured using Rat ELISA kit (Lianke, Hangzhou, China). The myeloperoxidase (MPO) production in astrocytes was accessed using Rat MPO ELISA kit (USCN, Wuhan, China). The optical density (OD) values at 450 nm and 570 nm were detected by ELX800 microplate reader (Biotek, Biotek Winooski, VT, USA).

Malondialdehyde (MDA) detection

The astrocytes were suspended in PBS and homog-

enized manually at low temperatures. The BCA protein concentration detection kit (Beyotime) was used to detect the total protein concentration of cell suspension. Subsequently, the MDA content was measured using MDA kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions.

Data analysis

Data analysis was performed with Graphpad Prism 8.0. The unpaired *t*-test was used to analyze differences between two groups. Statistical comparisons between multiple groups were analyzed by one-way ANOVA. All values are expressed as means ± standard deviation (SD). *P* < 0.05 was considered significant.

RESULTS

Lead exposure induced activation of astrocytes and autophagy in hippocampus

Sprague-Dawley rats were treated with lead acetate to establish a rat model of lead exposure. We first detected the expression and localization of LC3 in astrocytes by immunofluorescence. Our results showed that astrocytes were one of the major cells that undergo autophagy after lead acetate stimulation (Fig. 1A and Supplemental Fig. 1). Moreover, compared with the control group, the expression levels of GFAP and LC3 in astrocytes detected by immunofluorescence staining were increased by lead acetate in hippocampal tissues (Fig. 1A). The increased number of GFAP-positive cells was also observed in the lead acetate-treated group as compared with the control group (Fig. 1A). In addition, lead acetate treatment significantly elevated protein levels of LC3II and Beclin 1, whereas decreased p62 protein level in the hippocampus (Fig. 1B). Therefore, *in vivo* experiments showed that lead acetate treatment induced autophagy of astrocytes in the hippocampus and astrocyte activation.

Lead exposure induced autophagy in astrocytes

We next isolated astrocytes from hippocampal tissue of Sprague-Dawley rats; these cells exhibited > 98% positive staining for GFAP (Fig. 2A). The astrocytes were then treated with lead acetate (1 µM or 10 µM) for 24 hr. As shown in Fig. 2B, there were few autophagosomes or autophagolysosome-like structures in normal cells. However, under the stimulation of lead acetate, autophagosomes or autophagolysosomes appeared in the cells and increased in a dose-dependent manner. Furthermore, astrocytes treated with 10 µM lead acetate exhibited more autophagosomes than cells treated with 1 µM lead acetate (Fig. 2B). LC3II turnover assay is considered to be

Fig. 1. Lead exposure induced activation of astrocytes and autophagy in hippocampus. (A) The representative immunofluorescence images of GFAP (green), LC3 (red), and nuclei (blue), and the ratio of GFAP-positive staining in rat hippocampi treated with or without lead acetate. GFAP staining for astrocyte identification and DAPI for nucleus identification. Scale bar = 50 μ m. (B) The protein expression levels of LC3I, LC3II, Beclin 1, and p62 in rat hippocampi treated with lead acetate. $n = 6$. The data are expressed as mean $\pm$ SD. *** $P < 0.001$ compared with the control group.

the main method for detecting autophagic flux. We further detected the effects of lead acetate on autophagic flux by using LC3II turnover assay. Our results showed that the LC3II protein levels were further elevated in the presence of chloroquine (10 μ M) as compared with that in the absence of chloroquine after cells were treated with 1 μ M or 10 μ M lead acetate (Fig. 2C). Furthermore, compared with the control group, lead acetate treatment dose-dependently increased the protein levels of LC3II and Beclin 1, while p62 expression level detected by Western blot and immunofluorescence staining was significantly decreased in lead acetate-treated cells (Fig. 2D and 2E). Therefore, the results indicated that lead acetate treatment induced autophagy in astrocytes in a dose-dependent manner.

Lead exposure induced autophagy via blocking the Akt/mTOR pathway in astrocytes

We further explored the mechanism by which lead exposure induce astrocyte autophagy. The results showed that lead acetate treatment caused a dose-dependent decrease in p-Akt, p-mTOR, and p-p70S6K protein levels as compared with the control group (Fig. 3A). Akt/mTOR pathway negatively regulated autophagy. Collectively, the results indicated that lead exposure induced autophagy via negatively regulating the AKT/mTOR pathway in astrocytes.

Autophagy inhibitor 3-MA suppressed lead exposure-mediated inflammatory response and oxidative stress in astrocytes

Astrocytes were treated with lead acetate and/or 3-MA. The results of Western blot showed that LC3II protein level in the lead acetate group was much higher than

Fig. 3. Lead exposure induced autophagy via blocking the Akt/target of rapamycin (mTOR) pathway in astrocytes. (A) Relative protein expression levels of Akt/p-Akt, mTOR/p-mTOR, and p70S6K/p-p70S6K in astrocytes treated with or without lead acetate (1 μ M or 10 μ M). $n = 3$. The data are expressed as mean $\pm$ SD. ** $P < 0.01$ and *** $P < 0.001$ compared with the control group. # $P < 0.05$ and ## $P < 0.01$ compared with the lead acetate (1 μ M) group.

that in the control group, while LC3II expression level in the lead acetate group was significantly decreased by 3-MA (Fig. 4A). As presented in Fig. 4B and Fig. 4C, the expression levels of TNF- α and IL-1 β detected by ELISA assay were observably increased by lead acetate, whereas 3-MA treatment reversed lead acetate-induced TNF- α and IL-1 β secretion. Furthermore, the MDA and MPO levels in the lead acetate group were significantly up-regulated as compared with the control group (Fig. 4D and 4E). However, 3-MA treatment suppressed lead acetate-induced MDA and MPO production (Fig. 4D and 4E). Overall, the results demonstrated that lead acetate treatment triggered the inflammatory response and oxidative stress in astrocytes, which was reversed by 3-MA.

DISCUSSION

Here, we demonstrated that lead exposure triggers autophagy and further increased the inflammatory cytokines and oxidative stress in astrocytes. Astrocytes cover the entire central nervous system and participate in the regulation of diverse complex functions. Increased reactive astrocytes are an important marker of central nervous system diseases (Sofroniew and Vinters, 2010). Lead is a toxic substance that threatens human health. Lead is often considered a risk factor for neurodegenerative diseases. Studies have shown that lead exposure induced reactive astrocytes (Struzyńska *et al.*, 2001). GFAP is a marker of astrocyte activation. Chronic lead exposure led to astrocyte activation and

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Fig. 4. Autophagy inhibitor 3-MA suppressed lead exposure-mediated inflammatory response and oxidative stress in astrocytes. (A) Relative LC3I and LC3II protein levels in astrocytes treated with lead acetate (10 μ M) or/and 3-MA (5 mM). (B and C) The expression levels of IL-1 β and TNF- α in astrocytes treated with lead acetate or/and 3-MA were measured by ELISA assay. (D and E) The MDA and MPO production in astrocytes treated with lead acetate or/and 3-MA. $n = 3$. The data are expressed as mean $\pm$ SD. *** $P < 0.001$ compared with the control group. ## $P < 0.01$ and ### $P < 0.001$ compared with the lead acetate group.

increased GFAP expression level in the rat hippocampus (Selvín-Testa *et al.*, 1991; Stoltenburg-Didinger *et al.*, 1996). Similar to previous studies, the activation of astrocytes and the overexpression of GFAP were observed in the hippocampal tissues of rats treated with lead acetate. Furthermore, lead exposure often induces autophagy. In

particular, lead exposure induced autophagy in osteoblasts (Lv *et al.*, 2015). Moreover, lead exposure also induced autophagy in the hippocampus and further affected intellectual development and learning and memory (Zhang *et al.*, 2012). Here, we identified that GFAP-positive cells were increased in hippocampus and the autophagy-related

proteins were induced and overexpressed in hippocampus as well as in astrocytes after lead acetate treatment, suggesting that lead acetate treatment induced autophagy of astrocytes. In addition, we treated astrocytes with chloroquine, which effectively inhibits the fusion of autophagosome and lysosome and found that the accumulation of LC3II was further promoted in the presence of chloroquine as compared with that in the absence of chloroquine after cells were treated with lead acetate, suggesting that the accumulation of autophagosomes in astrocytes was mainly caused by the formation of autophagosomes (Liu *et al.*, 2015; Rubinsztein *et al.*, 2009). Overall, we demonstrated that lead exposure induces autophagy in astrocytes. Previous studies suggested that activation of autophagy induced astrocytes (Periyasamy *et al.*, 2016). Our results suggested that lead exposure may induce activation of astrocytes by triggering autophagy. We further explored the mechanism by which lead exposure induces astrocyte autophagy. The Akt/mTOR pathway is a negative regulatory pathway for autophagy. Studies have found that activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway inhibited autophagy induced by N-methyl-D-aspartate (Wang *et al.*, 2014). Besides, Xiao *et al.* (Lv *et al.*, 2015) explored the specific mechanism of osteoblast autophagy induced by lead exposure. The results showed that after lead exposure, autophagy was induced and mTOR and p70S6K phosphorylation levels were significantly reduced in osteoblasts, indicating that lead exposure induced osteoblast autophagy through mTOR pathway inhibition. Here, we found that the phosphorylation levels of Akt, mTOR, and p70S6K were significantly down-regulated in astrocytes treated with lead acetate, suggesting that the induction of astrocyte autophagy by lead exposure is achieved by blocking the Akt/mTOR pathway. Therefore, we demonstrated that lead exposure triggers astrocyte autophagy through the Akt/mTOR pathway and further induces the activation of astrocytes.

Activation of astrocytes is one of the potential ways of causing lead neurotoxicity. Astrocytes results in neuronal cell death by triggering the production of multiple cytokines and chemokines (Hostenbach *et al.*, 2014). Previous studies have found that lead exposure mediated inflammatory neurotoxicity via inducing the expression of the pro-inflammatory factor cyclooxygenase-2 (COX-2) in astrocytes (Wei *et al.*, 2014). Our results showed that lead exposure observably increased IL-1 β and TNF- α levels in astrocytes. TNF- α can induce glutamate secretion in astrocytes, which mediates the stimulating input of excitatory synapses (Santello *et al.*, 2011). Moreover, excessive TNF- α production disrupts the neural signaling network (Bezzi *et al.*, 2001). IL-1 β is a pro-

inflammatory factor, which has been widely of concern for its important role in the inflammatory response. High expression of IL-1 β can cause inflammatory neurotoxicity (Kempuraj *et al.*, 2016). Therefore, the release of IL-1 β and TNF- α in astrocytes may be a potential reason of neurotoxicity caused by lead. In addition, previous studies have found that neurotoxicity induced by lead exposure is also associated with oxidative stress (Villeda-Hernández *et al.*, 2001). Lead exposure causes the accumulation of lipid peroxidation products through induction of oxidative stress, including reactive oxygen species (ROS), MDA, and eventually causes toxicity (Sharma *et al.*, 2015). Here, we found that lead exposure caused MDA and MPO accumulation in astrocytes, suggesting that lead exposure induces oxidative stress in astrocytes. More importantly, the blockade of autophagy inhibited lead exposure-induced astrocyte oxidative stress and pro-inflammatory factor secretion. However, it is worth noting that 3-MA was not completely suppressed the lead mediated the secretion of pro-inflammatory factors and the accumulation of lipid peroxidation products in astrocytes, which may be due to insufficient concentration of 3-MA. The evidence is that the LC3II protein level in cells treated with lead acetate was not completely suppressed by 3-MA. The results indicated that autophagy mediates the release of inflammatory cytokines and oxidative stress in astrocytes induced by lead exposure.

Taken together, we demonstrate that lead exposure triggers autophagy in astrocytes by negatively regulating the Akt/mTOR pathway. Furthermore, autophagy mediates the activation of lead-induced astrocytes and further promotes the release of inflammatory cytokines and oxidative stress. Therefore, induction of autophagy in astrocytes may be the pathological basis of neurotoxic effects induced by lead exposure.

Conflict of interest---- The authors declare that there is no conflict of interest.

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