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Autophagy plays a protective role in Mn-induced toxicity in PC12 cells

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ABSTRACT

Excessive environmental or occupational exposure to manganese (Mn) is associated with increased risk of neuron degenerative disorders. Oxidative stress and mitochondrial dysfunction are the main mechanisms of Mn mediated neurotoxicity. Selective removal of damaged mitochondria by autophagy has been proposed as a protective mechanism against neuronal toxicant-induced neurotoxicity. Whether autophagic flux plays a role in Mn-induced cytotoxicity remains to be fully elucidated. The present study was designed to investigate the effect of Mn exposure on autophagy, and how modulation of autophagic flux alters the sensitivities of cells to Mn-elicited cytotoxicity. Rat adrenal pheochromocytoma PC12 cells were treated with Mn for 24 h to establish a cellular mode of Mn toxicity. Treatment of cells with Mn resulted in increased expression of autophagic marker LC3-II protein, as well as accumulation of p62, indicating an interference of autophagy flux caused by Mn. Pre-incubation of cells with antioxidant N-acetyl-L-cysteine (NAC) or resveratrol improved cell survival, accompanied by decreased LC3-II expression and increased expression level of p62, suggesting a down regulation of autophagy flux. To further determine the role of autophagy in Mn-induced cytotoxicity, the effect of chloroquine and rapamycin on cell viability was examined. Inhibition of autophagy flux by chloroquine exacerbated Mn-induced cytotoxicity, while induction of autophagy by rapamycin significantly reduced cell death caused by Mn. Furthermore, it was found that rapamycin, NAC and resveratrol improved cellular oxygen consumption accompanied by a decrease in cellular ROS generation and increase in GSH level, while chloroquine suppressed cellular respiration and deteriorated cellular oxidative stress. Collectively, these results demonstrate that autophagy plays a protective role in Mn-induced cell toxicity. Antioxidants NAC and resveratrol confer protective role in Mn toxicity mainly through maintaining mitochondrial dynamics and function, other than a modulation of autophagy flux.

1. Introduction

Manganese (Mn) is an essential trace element in maintaining normal cellular function, but excessive accumulation of Mn in the brain causes neurotoxicity. Chronic environmental and occupational exposure to Mn results in a progressive neurological disorder referred to as manganism, a syndrome closely resembling Parkinson's disease (Gorell et al., 1999; Lucchini et al., 2007; Olanow, 2004). Despite a great deal of efforts on the characterization of risk factors and understanding of the pathogenesis over last decades, the mechanism of Mn induced neurodegenerative damage remains unclear. Animal studies have revealed substantia nigra pars compacta as a main site of Mn accumulation in rat brain with vulnerability of dopaminergic neurons to Mn toxicity (Peres et al., 2016; Robison et al., 2015; Stanwood et al., 2009). Mn induced loss of dopaminergic neurons plays a critical role in the progressive development of manganism. Although the underlying mechanisms are not fully understood, mitochondrial dysfunction and oxidative stress are proposed to be key cellular events involved in Mn neurotoxicity (Smith et al., 2017).

Mn is an important catalytic cofactor of manganese-dependent superoxide dismutase 2, an important antioxidant enzyme localized in mitochondria. However, excess accumulation of Mn in mitochondria disrupts mitochondrial homeostasis and impairs mitochondrial function. Studies have well demonstrated that Mn inhibits mitochondrial complex I and II respiration as well as induces mitochondrial permeability transition (Malecki, 2001; Rao and Norenberg, 2004; Sriram et al., 2010). These effects of Mn on mitochondria directly result in the bioenergetic collapse that is particularly deleterious to neurons that

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intensely depend on intact mitochondrial respiration to maintain cellular function especially during neural activation (Erecinska and Silver, 1989; Hertz, 2008). In addition, inhibition of mitochondrial respiration may enhance electron leakage from the mitochondrial electron transfer chain, leading to overproduction of reactive oxygen species (ROS) that directly damage mitochondria, exacerbating mitochondrial and cellular oxidative stress, and leading to the release of cytochrome c triggering apoptotic cascade (Nishimura et al., 2001; Zorov et al., 2014). In this regard, a timely clearance of dysfunctional mitochondria induced by Mn may present a strategy for the cell to defend against or reduce the damage caused by Mn. Indeed, the cells have evolved a radical mechanism to remove “unhealthy mitochondria” through mitophagy, or mitochondrial autophagy, an autophagy-dependent process specifically to remove the energy producing organelle damaged or unwanted in the cell (Lemasters, 2005; Gomes and Scorrano, 2013).

Studies in recent years have implicated the regulation of autophagy in the development of neurodegenerative disorders including PD. Mitophagy in particular has been suggested to play an important role in neuroprotection mechanisms (Koentjoro et al., 2017; Son et al., 2012). However, autophagy is a dual-edged sword, where excessive activation of autophagy is involved in the mechanisms of chemical-induced toxicity as well as neurodegenerative disorders (Li et al., 2014). It has been reported that Mn treatment results in dysregulation of autophagy in dopaminergic neurons (Zhang et al., 2013). Yet, the exact role that autophagy plays in the Mn-induced neurodegeneration remains unclearly described. Further, antioxidants such as N-acetylcysteine (NAC), or resveratrol, a phytoalexin produced mainly from grapes, blueberries, raspberries, and mulberries, have been widely used as neuroprotective agents in manganese-induced neurotoxicity and neurodegenerative diseases (Gawlik et al., 2017; Rocha-González et al., 2008). Whether the effect of NAC or resveratrol on manganese neurotoxicity is involved in the regulation of autophagy is unknown.

The present study was conducted to determine whether and how the modulation of autophagy using both inhibitory and activation approaches of autophagy affect Mn toxicity in PC12 cells, a neuroendocrine cell line with the capability to produce the neurotransmitter dopamine (DA) and contain functional DA metabolism pathways. Furthermore, we investigated if regulation of autophagy is involved in the mechanism of NAC and resveratrol-mediated protection in Mn cytotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

$MnCl_2$, resveratrol, *N*-acetylcysteine (NAC), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rapamycin, chloroquine diphosphate, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Beijing Solarbio Science & Technology Co.,Ltd (Beijing, China). GSH, adenovirus Ad-GFP-LC3B, and antibody for β-actin were purchased from Beyotime Biotechnology (Beijing, China). Antibodies for LC3 and p62 were from Abcam (Cambridge, UK).

2.2. Cell line and cell culture

The rat pheochromocytoma neuro cell line PC12 was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C, 5% CO_2 in a humidified atmosphere.

2.3. Evaluation of cell viability

The cytotoxicity induced by $MnCl_2$ was evaluated by MTT assay, and was verified by sulforhodamine B (SRB) colorimetric assay, a widely used method for *in vitro* cytotoxicity assessment described previously (Vichai and Kirtikara, 2006). Cells (1.5×10^5 cells/well) were seeded onto 24-well microtiter plates and treated with Mn at various concentrations (0, 100, 200, 300, and 400 μM) for 24 h. Following the treatments, cells were exposed to MTT (0.1 mg/mL) for 4 h at 37 °C. The culture medium was then removed and the cells were solubilized into 2-propanol solution. Then, absorbance was measured at 570 nm. The viability of cells was expressed as a percentage of the absorbance measured in control cells. For SRB assay, cells were fixed with 10% (wt/vol) trichloroacetic acid and then stained with 4 mg/ml of SRB prepared in 1% (vol/vol) acetic acid for 30 min, after which the excess dye was removed by washing with 1% acetic acid five times. The protein-bound dye was dissolved in 10 mM Tris base solution and OD value was determined at 510 nm using a microplate reader. To observe the effects of NAC, resveratrol, rapamycin, and chloroquine on Mn toxicity, cells were pretreated with these chemicals at indicated concentrations for 2 h before exposure to 300 μM Mn for 24 h. Cell cytotoxicity was determined by MTT assay.

2.4. Measurement of cellular ROS generation

Intracellular ROS generation was estimated by flow cytometry utilizing a fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Solarbio, China). Cells (10^5 cells/well) were seeded onto 6-well culture plates and pre-treated with rapamycin, chloroquine, NAC, or resveratrol at the indicated concentrations for 2 h before exposure to 300 μM Mn for 12 h. Upon completion of the treatments, the cells were washed with phosphate buffered saline (PBS), and followed by incubation with serum-free medium containing 10 μM DCFH-DA for 30 min at 37 °C in the dark. The cells were then washed twice with PBS, trypsinized, and resuspended. The fluorescence intensity was analyzed using a Gallios™ Flow Cytometer (Beckman). Each determination of fluorescence was the mean fluorescence intensity of 30,000 cells.

2.5. Infection of ad-GFP-LC3B and imaging

PC12 cells were infected with adenovirus expressing GFP-LC3B fusion protein (Ad-GFP-LC3B, Beyotime Biotechnology, Beijing, China) at MOI 10. Cells were replaced with fresh medium 24 h after infection. Cells were pre-treated with rapamycin or chloroquine for 2 h, were then co-exposed to Mn (300 μM) for 12 h. At the end of the treatment, cells were washed three times with PBS, and fixed with 4% paraformaldehyde 10 min, then treated with 0.3%Triton for 10 min. DAPI was used for counterstaining of nuclei. The cells were observed under an inverted fluorescence microscope (Olympus IX73).

2.6. Measurement of cellular GSH/GSSG

The intracellular glutathione was measured using a GSH and GSSG Assay Kit from Beyotime as per the manufacturer’s instruction. Briefly, upon completion of the treatments, cells were harvested and washed with cold PBS. The cells were then mixed with 5% metaphosphoric acid, and lysed by two cycles of freezing and thawing. The samples were then centrifuged at 10,000*g* for 10 min, and the supernatant was collected for determination of total glutathione and oxidized GSSG. The intracellular glutathione was determined by measuring the absorbance at 412 nm using a microplate reader. The reduced GSH was obtained by subtracting GSSG from the total glutathione. The GSH/GSSG ratio was calculated to indicate cellular oxidative stress status.

2.7. Determination of oxygen consumption

Determination of mitochondrial respiration of PC12 cells was performed at 37 °C in a high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Austria). Measurements of oxygen consumption were