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Cadmium induced inhibition of autophagy is associated with microtubule disruption and mitochondrial dysfunction in primary rat cerebral cortical neurons

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ABSTRACT

Recent studies have reported that mitochondria serve as direct targets for cadmium- (Cd-) induced neuronal toxicity, which can be attenuated by autophagy. The molecular mechanisms' underlying Cd-induced mitochondrial dysfunction and autophagy in neurons are not known. In this study, we studied the upstream signaling pathways induced by Cd-mediated mitochondrial metabolism alterations using primary rat neuron as a model. We found that Cd induced the destruction of microtubules (MTs), and resulted in tau hyper-phosphorylation and decreased acetylated tubulin levels, which were related to a decrease in mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP) levels. As a result of taxol disruption, alterations in macroautophagy, like altered cellular distribution of the autophagy-related protein light chain 3 beta (LC3B) and the expression of Atg5 were found compared with Cd group. We found for the first time that MT disruption induced by Cd reduced the levels of autophagy, leading to mitochondrial dysfunction. These observations suggest new therapeutic strategies aimed to activate or ameliorate pro-survival macroautophagy.

1. Introduction

Cadmium (Cd) is an occupationally and environmentally relevant toxic element. The half-life of Cd in the human body is more than 15 years, and it has been reported that the accumulation of Cd within the human body is likely to increase in the future, which may result in a higher incidence of Cd-related diseases, including carcinogenesis, immune-depression and neurodegeneration (Kim et al., 2005). Moreover, Cd added extracellularly is taken up by cells and is targeted to the mitochondria, where it exerts further toxicity (Chuang et al., 2000), which is a notable feature of Alzheimer's disease (AD) (Cardoso et al., 2010; Santa-Maria et al., 2005). Changes in mitochondrial metabolism are key events in Cd-induced neurodegenerative disorders related to neuronal disturbances and may include activation of autophagy. Autophagy, which is a lysosomal pathway for the turnover of organelles and long-lived proteins, is a key determinant of cell survival and longevity. Larsen and Sulzer demonstrated that the activation of autophagy and mitochondria plays a key role in neuronal cell survival (Larsen and Sulzer, 2002). Apart from being central in the regulation of cell death, mitochondria can also provide the cell with adenosine triphosphate (ATP). During macroautophagy, an elongated "isolation" membrane sequesters a region of cytoplasm to form a double-membrane-limited autophagosome. The knowledge that autophagic insufficiency may compromise cellular degradation mechanisms and may culminate in the progressive accumulation of dysfunctional mitochondria, the buildup of aberrant protein aggregates and result in a lysosomal burden shields give us new insight into the way we address AD (Cardoso et al., 2010).

Autophagy is a tightly regulated process. There are normally and steadily low basal levels of autophagy in the brain. This pathway is up-regulated under toxic stress or with the formation of misfolded proteins (Eskelinen, 2008), resulting in decreased mitochondrial function and a strong autophagic response. Recently, it was demonstrated that autophagy is constitutively active in neurons and is required for survival (Larsen and Sulzer, 2002). The mammalian autophagic marker light chain 3 (LC3) is an interactive partner of microtubule- (MT-) associated protein (Schoenfeld et al., 1989), which is functionally redundant with regard to autophagosome formation and selective autophagy are regulated by LC3 (Perrino and Chou, 1986). It has been reported that LC3II-associated with mature autophagosomes move along microtubular tracks, and fuse with lysosomes to form autolysosomes in which LC3II is degraded along with the cargo of the autophagosome (Kim et al., 2007).

MTs are polymers of tubulin dimers whose dynamics are regulated by MT-associated proteins. Tubulin is a major structural protein in

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neurons that polymerizes to form MTs (Furukawa and Mattson, 1995). Tubulin constantly polymerizes and depolymerizes to facilitate trafficking of organelles along microtubular tracks and chromosomal segregation in mitosis (Desai and Mitchison, 1997; Downing, 2000). Sequestosome 1 (p62/SQSTM1), which is the best characterized autophagy substrate in mammals, localizes at the autophagosome formation site and directly interacts with LC3 through the LC3-interacting region. Moreover, LC3, Atg5 and Atg7 are essential genes for mammalian macroautophagy (Nishida et al., 2009). It is subsequently incorporated into the autophagosome and finally degraded. Many studies have documented (Maruyama et al., 2014) that changes in axonal transport due to a reduction in the assembly of MTs play an important role in the pathogenesis of AD (Santa-Maria et al., 2005).

The exact mechanism by which Cd induces cell death remains unclear. Yuan et al. had reported that extracellular Cd can act intracellularly to aggravate mitochondrial dysfunction (Yuan et al., 2013). So far, no data have been available concerning the activation of autophagy and the performance of the MT network underlying it in neurons. Therefore, we hypothesized that the accumulation of MT instability triggered by Cd decreased autophagy levels, which caused mitochondrial dysfunction in neurons.

2. Materials and methods

2.1. Reagents and chemicals

Fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA); ATP analysis kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Rhodamine- (TRITC-) conjugated anti-rabbit immunoglobulin G (IgG) was from Abcam Technology (USA). The radio-immunoprecipitation assay (RIPA), Solarbio lysis buffer, anti-LC3 polyclonal antibody, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), Neurobasal® medium, B-27® supplement, 4′,6-diamidino-2-phenylindole (DAPI) stain, Dulbecco's modified Eagle's medium (DMEM)-F12 and LC3 antibody were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other antibodies were purchased from Cell Signaling Technology (CST, USA).

2.2. Cell isolation and culture

Fetal Sprague–Dawley rats at 18–19 days of gestations were obtained from the Laboratory Animal Center of Jiangsu University (Zhenjiang, China). Primary rat cerebral cortical neurons were cultured from fetal Sprague–Dawley rats as described by Yuan et al. (Kochl et al., 2006), with minor modifications. Briefly, neonatal brain tissues were removed from rats and placed on 35 mm glass petri dishes containing 2 mL ice-cold D-Hanks. The cortex was cut into tiny particles and digested with trypsin for 20 min at 37 °C. The cortex was easily dissociated by triturating in DMEM/F12 containing 10% fetal calf serum, and using a sterile, long-neck Pasteur pipette. The cells were plated into 6-well plates at a density of 1×10^6 cells/well in Neurobasal® medium supplemented with 2% B-27®, 100 mg/L poly-L-lysine, 1 mmol/L L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin, and cultured at 37 °C in 5% CO_2. On Day 3, the cultures were incubated with 2.5 mg/L cytosine arabinoside for 24 h to suppress the growth of glial cells. The medium was replaced every 3 days; after replacing the medium twice, the cells were used for the experiments. Cell viability was assessed by the trypan blue exclusion, and it was routinely higher than 95%. Cell purity was checked by staining cells with toluidine blue to identify neurons. This study was carried out in strict accordance with the recommendations prescribed in the Guide for the Care and Use of Laboratory Animals of the National Research Council. Rat were housed in controlled conditions of lighting (12 h light/dark cycles), temperature (24 ± 2 °C) and were provided with standard food and water. The Animal Care and Use Committee of Yangzhou University approved all experiments and procedures conducted on the animals (approval ID: SYXK (Su) 2007–0005).

2.3. Cell viability assay

Cells were seeded at a density of 2×10^4 cells/well in 96-well plates. Cells were treated with 20 μmol/L Cd for 0–24 h at the designated time points; cell viability was measured by the MTT assay, which is based on the conversion of the tetrazolium salt to the colored product, formazan. In brief, 20 ml MTT solution (5.0 g/L in PBS) was added into each well of the 96-well plates (containing 100 ml medium and cells) 4 h before the end of incubation. The supernatant was then discarded, and 150 ml DMSO was added to dissolve the formazan. The absorbance was measured at 570/630 nm by the microplate reader (Sunrise, Austria).

2.4. Confocal microscopy visualization of LC3 beta (LC3B) and tubulin

Neurons were treated with 20 μmol/L Cd with or without 5 nmol/L taxol for 12 h. After treatment, neurons were washed twice with phosphate-buffered saline (PBS) and fixed for 30 min at 4 °C using 4% paraformaldehyde. The fixed cells were washed again with PBS, permeabilized with 0.2% TritonX-100 and blocked with 3% bovine serum albumin (BSA) which was purchased from Solarbio Life Science. The permeabilized cells were incubated with primary antibody (1:500 anti-alpha-tubulin or 1:200 anti-LC3B). Afterwards, cells were incubated for 1 h with the appropriate secondary antibody.

2.5. ATP quantification

The harvested cells were homogenized in ice-cold physiological saline in an ultrasonic disintegrator. The cell homogenates were centrifuged at 10,000 *g* for 10 min and supernatants were obtained. The concentration of the protein in the supernatant was determined by the Folin phenol method. The concentration of ATP was assayed in accordance with the ATP detection protocol.

2.6. Assay of the mitochondrial membrane potential

To measure the mitochondrial membrane potential (MMP) the lipophilic cationic probe JC-1 were used. JC-1 emits light at red and green wavelengths according to the concentrations absorbed by the mitochondria: at a normal membrane potential, JC-1 forms aggregates that emit an orange–red fluorescence, but decrease in the MMP values result in increases of the JC-1 monomer, which then emits a green fluorescence. Therefore, the red and green fluorescence of JC-1 reflects changes in the MMP of the mitochondrial membrane. The cells were harvested, washed twice with PBS, incubated with JC-1 (1:200, diluted with PBS) for 30 min at 37 °C and analyzed on a BD-FACSAria™ flow cytometer. The MMP was assessed based on the fluorescence intensity of JC-1, which was recorded at an excitation wavelength of 490 nmol/L and an emission wavelength of 590 nmol/L.

2.7. Western blot analysis

Western blotting was performed as described previously by Yuan et al. (Yuan et al., 2013), with 40 μg protein loaded for every sample. Equal amounts of protein were separated by 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking in 5% fat-free milk for 2 h, the membranes were incubated overnight at 4 °C with antibodies against LC3, p62, tau, phosphor-tau, acetylated-tubulin, beclin-1 and β-actin (1:1000 dilution). Detection was performed with the appropriate horseradish peroxidase- (HRP-) conjugated secondary antibodies (1:5000 dilution) and enhanced chemiluminescence reagent in order