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Oxidative stress involvement in manganese-induced alpha-synuclein oligomerization in organotypic brain slice cultures

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ABSTRACT

Overexposure to manganese (Mn) has been known to induce neuronal damage. However, little is known of the role that reactive oxygen species (ROS) play in protein aggregation resulting from Mn exposure. The current study investigated whether oxidative stress is involved in manganese-induced alpha-synuclein oligomerization in organotypic brain slices. After application of Mn (0–400 μM) for 24 h, there was a dose-dependent increase in average percentage of propidium iodide positive (PI^+) nuclei in slices and levels of lactate dehydrogenase (LDH) in the culture medium. Moreover, the treatment with Mn resulted in a dose-dependent increase in neurocyte apoptosis, ROS level, and decrease in superoxide dismutase (SOD) activity. Mn also caused oxidative damage in cell lipid and protein. At the same time, the exposure of Mn leaded to significantly increase in the expression of alpha-synuclein mRNA and protein. Alpha-synuclein oligomerization occurred in Mn-treated slices, especially on membrane-bound form. It indicated that alpha-synuclein oligomers were more likely to combination cell membranes and resulting in membrane damage. Mn-induced neurocyte damage and alpha-synuclein oligomerization were also partially alleviated by the pretreatment with GSH and aggravated by H_2O_2 pretreatment. The findings revealed Mn might exert its neurotoxic effects by oxidative stress-mediated alpha-synuclein oligomerization in organotypic brain slices.

1. Introduction

Manganese (Mn) is an essential nutrient, important in the biochemical reactions of several enzymes including manganese-dependent superoxide dismutase. It plays an important role in iron metabolism, and both are required for proper brain function (Roels et al., 2012). However, exposure to high concentrations of Mn is known to result in neurotoxicity. Cases of manganese intoxication have occurred worldwide for almost two centuries, causing a severe, debilitating neurological disease resembling Parkinson's disease (PD) referred to as manganism. Chronic exposure to Mn produces a parkinsonian syndrome, particularly in miners, welders, and ferroalloy and battery manufacture workers (Criswell et al., 2012). Several studies have also indicated that some heavy metals, such as Mn, increase the risk of PD (Guilarte, 2010a). In China, accumulation of Mn and Fe via unknown routes might be involved in the etiology of PD in the general population (Fukushima et al., 2010). Therefore, understanding the exact molecular mechanisms of Mn toxicity may play a critical role linking environmental neurotoxins to the pathogenesis of PD.

The current literature suggests that no single mechanism can explain the multitude of effects observed in Mn-induced neurotoxicity. The mechanisms involved in the Mn-induced neurotoxicity are mainly related to induction of oxidative stress, alteration of neurotransmitter metabolism and calcium homeostasis (Racette et al., 2012). Oxidative stress and apoptosis have been actively investigated as neurotoxic mechanisms over the past two decades, resulting in a greater understanding of neurotoxic processes. Nevertheless, emerging evidence indicates that protein aggregation is also one of the important cellular and molecular correlates of neurodegenerative diseases resulting from chronic Mn exposure.

Alpha-synuclein is a highly charged 140-amino acid heat stable protein that is soluble and natively "unfolded". It is predominantly expressed in neurons of the central nervous system (CNS), where it localizes to presynaptic terminals in close proximity to synaptic vesicles. Alpha-synuclein is a protein with natural tendency to aggregate into oligomers and is considered to be a key player in the pathophysiology of PD. The evidence suggests that early intermediary oligomers, rather than mature fibrils of alpha-synuclein, are the pathogenic species (Park et al., 2011). Its overexpression

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promotes apoptotic cell death in a variety of cell lines and animal models (Dawson and Dawson, 2003). The early oligomeric intermediates are assumed to be very toxic to the cell and are able to induce leakage in vesicles (Wan and Chung, 2012). Although several studies have reported that Mn could induce alpha-synuclein overexpression (Cai et al., 2010; Guilarte, 2010b; Peneder et al., 2011), little data exist on oxidative stress involved in Mn-induced alpha-synuclein oligomerization, information that is critical for more fully evaluating the Mn-induced neurotoxicity. Therefore, we will observe the effect of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) on Mn-induced alpha-synuclein oligomerization.

2. Materials and methods

2.1. Chemicals

Manganese (II) chloride tetrahydrate, GSH, propidium iodide (PI), 2′,7′-dichlorofluorescein diacetate (DCF-DA), 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), Protein carbonyl and thiol detection reagent kits were from Nanjing Jiancheng Biotech. Co. Ltd. (Cat No: A020-2, A001-3, A003-4, A087 and A063-2; China). *PrimeScript*® RT Enzyme Mix I and SYBR® *Premix Ex Taq*™ II kit was from TaKaRa Biotech. Co. Ltd. ProteoExtract® Subcellular Proteome Extraction Kit was obtained from EMD Millipore (Cat. No. 539790; Germany). Polyacrylamide gradient gel electrophoresis (4–20%) was obtained from Dycent Biotech Co. Ltd. (Cat No: B1611105; China). Rabbit alpha-synuclein primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals of analytical grade were obtained from local chemical suppliers.

2.2. Preparation of organotypic slice cultures

Organotypic slice cultures were prepared according to the methods described previously (Stoppini et al., 1991; Ohnishi et al., 2010). Briefly, Wistar rats at postnatal days 3–4 were provided by the Laboratory Animal Center of China Medicine University (SPF grade, production license No. SCXK2008-0005). After dissection of the brain and removal of the frontal and occipital poles (including the cerebellum) the specimens were placed into Hank's balanced salt solution (HBSS, Invitrogen) and kept at 4 °C and pH 7.35. The specimens were sliced in 300 μm thick sections on a NVSL/NVSLM1 tissue slicer (World Precision Instruments Inc., USA). The first slices (in most cases not more than four) were abandoned until the basal ganglia displayed its typical cytoarchitecture. Normally 4–6 slices with intact basal ganglia cytoarchitecture were collected, transferred to cell culture inserts (pore size 0.4 μm, Falcon, Millipore, Bedford, MA), placed in 6-well culture dishes (Falcon) and fed with 1 mL culture medium consisting of 50% minimum essential medium, 24% horse serum and 25% HBSS, 1% penicillin-streptomycin (all from Invitrogen) and supplemented with 36 mM glucose, and 25 mM Hepes (Sigma, St. Louis, MO, USA) (pH 7.2). Cultures were maintained at 37 °C under room air + 5% CO_2. After 1 day in culture, culture medium was replaced with fresh medium containing no antibiotics. The animal experiment was carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the local authorities. All efforts were made to minimize the number of animals used and their suffering.

2.3. Drug treatment

Cultured slices at 13–15 days in vitro were incubated for 24–48 h in serum-free medium, where minimum essential medium/Hepes substituted for horse serum. Then, slices were exposed to Mn (0, 25, 100, 400 μM) for 24 h and GSH (2 mM), or H_2O_2 (100 μM) dissolved in serum-free medium. For GSH or H_2O_2 supplementation experiments, cultured slices were maintained in these media at 37 °C, 5% CO_2 for 12 h before treatment with Mn.

2.4. Slice injury assessment

To estimate the cell injury of the basal ganglia region, the percentage of propidium iodide positive (PI^+) nuclei was assessed after 24 h of treatment with Mn by counting the number of PI and DAPI-positive nuclei using established procedures (Ebrahimi et al., 2010). PI and DAPI fluorescence were observed with a confocal laser scanning microscopy (FV 1000S-IX81, Olympus, Japan) equipped with a 40× objective lens. Fluorescence images were captured through a CCD camera, with the use of FV10-ASW software (version 03.00.01.15). LDH release is also an indicator of the integrity of cell membrane in that LDH releases from cells after cells were injured. After the treatments, LDH releases in the medium were measured according to the methods as previously described (Xu et al., 2009). The values of absorbance were read at 440 nm by the use of the microplate reader (Bio-Rad, USA) and the results of the absorbance of the test wells were expressed as percent of the control wells. Results of an individual experiment that reflect similar data obtained on at least four separate occasions.

2.5. Determination of ROS formation in basal ganglia

ROS formation was monitored using the oxidation-sensitive fluorescent dye DCFH-DA, as described previously (Cheng et al., 2011). Briefly, cells (1×10^6) were suspended in PBS and incubated with 10 μM DCF-DA at 37 °C for 30 min. The fluorescence increase, which is due to the hydrolysis of DCF-DA to DCF by nonspecific cellular esterase and its subsequent oxidation by peroxides, was measured and monitored at 488 nm (excitation)/525 nm (emission) by a FACScan flow cytometer (Becton Dickinson, Germany). The results are expressed as fluorescence compared with controls.

2.6. Oxidative damage assays

SOD and MDA determined spectrophotometrically in the homogenized with the methodology according to the manufacturer's introduction. SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was measured by the degree of inhibition of this reaction. Absorbance differences were recorded at 560 nm for SOD. MDA was measured as the production of lipid peroxide, which in combination with thiobarbituric acid (TBA) forms a pink chromogen compound whose absorbance at 530 nm was recorded. The total protein determination was performed according to the procedure of Lowry et al. (1951) using BSA as the standard.

2.7. Protein carbonyl and thiol contents of alpha-synuclein assay

Protein carbonyl and thiol contents were measured as the indicators of alpha-synuclein oxidative damage. Brain slices were scraped in buffer containing 20 mM Tris, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA (pH 7.0), and protease inhibitors (200 mM PMSF and a commercial protease inhibitor cocktail). Cell suspensions were centrifuged at 12,000 × *g* for 15 min at 4 °C. Supernatants were removed and stored at −80 °C. The protein concentration of each sample was determined by with the BCA reagent. 500 μg of cell lysates was incubated with primary antibody (1:50 alpha-synuclein, Santa Cruz, CA), overnight at 4 °C, and with gentle agitation. Lysates were then incubated with 100 μl of protein-A/G beads for 2 h at 4 °C and with gentle agitation. After completing this incubation, lysate tubes were centrifuged at 65 × *g* for 5 min at 4 °C, the supernatant was removed, and the beads were washed in the previously described buffer 7 times (each time centrifuging at 4 °C and removing the supernatant). For the two first washes, the buffer was supplemented with 1% Triton X-100. For the next three washes, the buffer was supplemented with 1% Triton X-100 and 500 mM NaCl. The two final washes were performed with unsupplemented buffer. Finally, the last supernatant was removed and 25 μl of 2× sample buffer was added. The sample was boiled at 95–100 °C for 5 min to denature the protein and to separate it from the protein-A/G beads. The boiled proteins were centrifuged at 12,000 × *g* for 15 min at room temperature, and the supernatants were collected.

Protein carbonyl contents were detected by the reaction with 2,4-dinitrophenylhydrazine (DNPH) method according to the manufacturer's introduction. Briefly, the DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) TCA and washed three times with 2 ml of an ethanol/ethyl acetate mixture (1:1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution. The absorbance was measured at 370 nm. Protein thiol contents were detected by the reaction with 5,5′-dithio bis-(2-nitrobenzoic acid) (DTNB) method according to the manufacturer's introduction. The colorimetric reaction was measured at 412 nm. Protein thiol contents were calculated by total thiol subtracting non-protein thiol.

2.8. Total RNA isolation and quantitative real-time PCR analysis

For total RNA isolation, slices were homogenized in RNAiso Plus. After 5 min room temperature incubation (20 °C), chloroform was added for phase separation. The upper aqueous phase was collected and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol and was air-dried. It was finally redissolved in RNase-free water. The absorbance of the RNA solution was determined using NanoPhotometer (IMPIEN, Germany) at 260 and 280 nm, respectively. A260/A280 ratios were between 1.6 and 1.8.

The first strand cDNA was synthesized from 1 μg of total RNA by Reverse Transcriptase using PrimeScript® RT Enzyme Mix I (TaKaRa) and oligo (dT) primers (TaKaRa) according to the manufacturer's protocol. Real-time quantitative PCR (RT-PCRq) was performed by SYBR® *Premix Ex Taq*™ II kit (TaKaRa) using ABI 7500 Real-Time PCR System (Applied Biosystems, USA). Two microliters of template cDNA were added to the final volume of 20 μl of reaction mixture. Real-time PCR cycle parameters included 30 s at 95 °C followed by 40 cycles involving denaturation at 95 °C for 5 s, annealing at 60 °C for 34 s and elongation at 72 °C for 20 s. The following primers were used: for alpha-synuclein (Paillusson et al., 2010), forward: 5′-CACAAGAGGGAATCCTGGAA-3′; reverse: 5′-TCATGCTGGCCGTGAGG-3′; for β-actin, forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′; reverse: 5′-GACTCATC-GTACTCCTGCTTGCTG-3′ Expressions of alpha-synuclein was corrected to β-actin