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Mitochondrial proteomic alterations caused by long-term low-dose copper exposure in mouse cortex

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HIGHLIGHTS

- Copper caused abnormal expression of mitochondrial proteins in brains.
- Functional categories of the proteins affected by copper were revealed.
- GRP75/GRP78 may be the key players in copper neurotoxicity.

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ABSTRACT

Mitochondrial dysfunction is involved in neurotoxicity caused by exposure of various chemicals such as copper. However, the effects of long-term low-dose copper exposure on mitochondrial proteome remain unclear. In this study, we found the treatment of copper (0.13 ppm copper sulfate in drinking water) for 12 months caused abnormal expression of a total of 13 mitochondrial proteins (7 up-regulated and 6 down-regulated) as revealed by two-dimensional electrophoresis coupled with mass spectrometry in mouse cortex. Protein functional analysis revealed that these differentially expressed proteins mainly included apoptosis-associated proteins, axon guidance-associated proteins, axonogenesis-associated proteins and mitochondrial respiratory chain complex. Among these differentially expressed mitochondrial proteins, GRP75 (75 kDa glucose-regulated protein) and GRP78 (78 kDa glucose-regulated protein) were found to be significantly down-regulated as confirmed by Western-blot analysis. The down-regulation of GRP75 was shown to promote apoptosis. The down-regulation of GRP78/BiP could up-regulate endoplasmic reticulum (ER) stress mediators and thus cause apoptosis. Our study suggested that these differentially expressed mitochondrial proteins such as GRP75 and GRP78 could be involved in neurotoxicity caused by long-term low-dose copper exposure and serve as potential molecular targets for the treatment of copper neurotoxicity.

1. Introduction

Copper (Cu) is one of essential elements. This trace metal is necessary for normal physiology in all organisms, serving as a cofactor in several metalloproteins required for critical cellular functions such as aerobic metabolism (Pena et al., 1999). Copper is an important component of multiple proteins and enzymes, including those that participate in the generation of high energy metabolites in mitochondria (Brewer, 2010). Copper, a catalytic cofactor of cytochrome c oxidase, is vital for aerobic metabolism.

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However, copper has been reported to evoke multiple adverse reactions at abnormally high concentrations leading to cellular dyshomeostasis and cell death (Pena et al., 1999).

A body of evidence has shown that mitochondria are sensitive to copper in endotherms and ectotherms contributing to impairment of oxidative phosphorylation (Saris and Skulskii, 1991). Notably, there is substantial empirical evidence that the structure and function of mitochondria are disrupted by the toxicity of copper (Krumschnabel et al., 2005). A previous study reported that copper could increase mitochondrial generation of reactive oxygen species (ROS) and exacerbate the damage to the mitochondria (Zafar et al., 2016). In addition, some studies demonstrated that copper exposure of a low dose (0.13 ppm) caused spatial memory impairment of mice, and exerted significant toxic effects on the brains of mice (Singh et al., 2013), suggesting that the toxic effects of copper were closely linked with mitochondrial impairment. However, the impact of copper exposure especially low-dose copper exposure on the expression of mitochondrial proteins remains unclear.

In this study, we investigated the effects of long-term low-dose copper exposure on the expression of mitochondrial proteome in mouse cortex by using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with MALDI-TOF-MS/MS. We identified the potential key molecules which may be involved in neurotoxicity caused by copper exposure.

2. Materials and methods

2.1. Animals and treatment

The mice (strain: B6129SF2/J) were purchased from Jackson Laboratory. Animal treatment and care procedures were carried out following the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised in 1985) and the Regulations for Animal Care and Use from the Committee of the Experimental Animal Center at Shenzhen Center for Disease Control and Prevention. 3-month-old mice were gave the drinking water with 0.13 ppm copper (cupric chloride, Sigma) for a duration of 12 months. The concentration of the copper is equivalent to 1/10 of the level that the United States environmental protection agency (EPA) allows in drinking water (1.3 ppm). All the mice were housed in groups of 15 mice per cage ($470 \times 350 \times 200$ mm) with free access to food and water, and maintained on a 12-h light-dark cycle with the light on from 6:00 am to 6:00 pm at stable temperature (20 ± 2 °C) and humidity (55 ± 5%). All efforts were made to minimize suffering of the animals and to reduce the number of mice used.

2.2. Isolation of mitochondria

Copper-exposed mice and the control mice were euthanized. The cortex was isolated on an ice-cold plate and then stored at −80 °C. Mitochondria were isolated by using the mitochondria isolation kit for tissue (Thermo Scientific, USA). All the steps were carried out at 4 °C. Briefly, the cortex was homogenized in 300 mL 1×PBS for 10 min by using a motor-driven tissue grinder (Sangon Biotech, G506003), followed by centrifugation at 1,000*g* for 5 min at 4 °C. The pellet was suspended in 800 μL of BSA/reagent A solution and incubated on ice for exactly 2 min. Then 10 μL mitochondria isolation reagent B was added to the suspending liquid and incubated on ice for 5 min. Subsequently, 800 μL mitochondria isolation reagent C was added to the tube, then centrifuged at 700*g* for 15 min at 4 °C. Finally, the supernatant was transferred to a new tube and centrifuged at 12,000*g* for 15 min at 4 °C. The mitochondrial pellets were stored at −80 °C until used.

2.3. Protein sample preparation

Mitochondrial pellets were suspended in DIGE-specific lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris–HCl, 4% CHAPS, pH 8.5) and incubated on the ice for 30 min. Subsequently, cortical mitochondrial samples were centrifuged at 20,000*g* at 4 °C for 60 min, then the supernatant was centrifuged at 15,000 g at 4 °C for 30 min to remove salt and other impurities. The protein solution was collected and the protein concentration was determined using a 2-D Quant Kit (GE Healthcare, USA) in accordance with the manufacturer's protocol.

2.4. DIGE labeling of mitochondrial proteins

In order to achieve a final dye concentration of 1 nmol/L as stock solution, each vial of CyDye (GE Healthcare, USA) was reconstituted in 99.8% anhydrous *N*,*N*-dimethylformamide (DMF, Sigma 227056). A working solution of 200 pmol/μL of each CyDye was used to label the proteins. All the samples from the mice were diluted to 5 μg/μL after the protein quantification. 5 μL of each protein sample (25 μg) was minimally labeled with 200 pmol of either Cy3 (GE Healthcare, 25-8008-61) or Cy5 dyes (GE Healthcare, 25-8008-62). In addition, a mixture of all mitochondrial samples (25 μg each) was labeled with Cy2 dye to make an internal standard. The labeling reaction was incubated on ice in the dark for 30 min. Subsequently, the reaction was quenched by the addition of 10 mM lysine (Sigma, L5626) for 10 min at 4 °C in the dark. After labeling, the Cy2-, Cy3- and Cy5-labeled samples were mixed together.

2.5. 2-D difference gel electrophoresis (2D-DIGE)

The first dimension was performed on Ettan IPGphor Isoelectric Focusing System (GE Healthcare). The equal volume of 2 × lysis buffer (8 M urea, 2% CHAPS, 0.2% DTT, 2% (v/v) IPG buffer pH 3–11 NL, 0.002% bromophenol blue) was added to each mixture labeled samples and incubated on ice for 10 min. Then rehydration buffer was added to make the total volume of the sample up to 450 μL. Equal amounts of labeled samples (75 μg) were put into 24 cm pH 3–11 NL immobiline Dry Strips (GE Healthcare). Then 2 mL of mineral oil was added to cover each strip to reduce solvent evaporation. The proteins were taken up into the strips by active rehydration at 50 V for 18 h. The conditions for IEF were step 300 V for 12 h, step 500 V for 2 h, step 1000 V for 2 h, gradient 8000 V for 8 h, step 8000 V for 8 h at 20 °C and the temperature of the room was kept at 18 °C. The focused strips were stored at −80 °C until the second dimension of gel electrophoresis was performed.

2.6. SDS-PAGE

After IEF, the focused strips were equilibrated with a reducing equilibration buffer containing 30% (v/v) glycerol, 6 M urea, 75 mM Tris–HCl buffer (pH 8.8), 2% (w/v) SDS and 1% (w/v) DTT for 15 min at room temperature on the shaking table Subsequently, the strips were re-equilibrated in the buffer containing 30% (v/v) glycerol, 6 M urea, 75 mM Tris–HCl buffer (pH 8.8), 2% (w/v) SDS and 4.5% IAA. The equilibrated strips were loaded on the top of 12.5% SDS-PAGE gels with 0.5% (w/v) ultra low melting point agarose sealing solution (25 mM Tris, 192 mM glycine, 0.1% SDS, 0.5% (w/v) agarose, 0.02% bromophenol blue). Electrophoresis was performed using an Ettan DALTsix electrophoresis system (GE Healthcare) with the following conditions: 1 W/gel for 1 h, subsequently 10 W/gel for 5 h in the dark at 15 °C. The gels were immediately scanned using a Typhoon TRIO Variable Mode Imager (GE Healthcare). In order to control for variation in the signal across gels, the PMT was set to