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Cerebral ganglion ultrastructure and differential proteins revealed using proteomics in the aplysiid (Notarcus *leachii cirrosus* Stimpson) under cadmium and lead stress

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ABSTRACT

Cadmium (Cd) and lead (Pb) are both highly toxic metals in environments. However the toxicological mechanism is not clear. In this study, the aplysiid, Notarcus *leachii cirrosus* Stimpson (NLCS) was subjected to Cd (NLCS-Cd) or Pb (NLCS-Pb). The cerebral ganglion of NLCS was investigated with a transmission electron microscope. Next the differential proteins were separated and identified using proteomic approaches. Eighteen protein spots in NLCS-Cd and seventeen protein spots in NLCS-Pb were observed to be significantly changed. These protein spots were further excised in gels and identified. A hypothetical pathway was drawn to show the correlation between the partially identified proteins. The results indicated that damage to the cerebral ganglion was follows: cell apoptosis, lysosomes proliferation, cytoskeleton disruption, and oxidative stress. These phenomena and data indicated potential biomarkers for evaluating the contamination levels of Cd and Pb. This study provided positive insights into the mechanisms of Cd and Pb toxicity.

1. Introduction

The pollution of aquatic ecosystems with heavy metals is a serious threat to the environment (Ling et al., 2009). These heavy metals are not easily degraded and are accumulated through the food chain (Mazzei et al., 2014). The level of heavy metal pollution in aquatic ecosystem is usually monitored by chemical methods such as atomic absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS). These measurements of metal concentration provide useful information, but they do not show direct evidence of bioavailability, bioaccumulation, or toxicity of heavy metals to aquatic organisms (Ling et al., 2009). Thus, identified proteins can be used for monitoring the toxic concentrations of heavy metals (Vieira et al., 2015).

Various heavy metals such as cadmium (Cd) and lead (Pb) are environmental and industrial pollutants and are extremely toxic metals which can be accumulated in the human body and cause severe damage to organs including the nervous system. Cd and Pb are non-functional heavy metals placed under the category of environmental pollutants due to their toxic effects on plants, animals, and humans (Ewa et al., 2015). Cd can easily cross the brain parenchyma, which may lead to reduced attention span, olfactory dysfunction, and memory deficits in individuals exposed to it (Wang et al., 2015). And Cd is a possible etiological factor of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Okuda et al., 1997). It has been reported that Cd can induce neuronal apoptosis (Yuan et al., 2015). Increasing evidences demonstrate that Pb also has neurotoxicity (Toscano and Guilarte, 2005). Pb is a strong toxicant for the development of central nervous system in children and animals. Pb exposure has been found to impair learning and memory in animals via affecting the synaptic functional plasticity (Xiao et al., 2014).

Abbreviations: NLCS, Notarcus *leachii cirrosus* Stimpson; AAS, atomic absorption spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; Cd, cadmium; Pb, lead; 2D-PAGE, two dimensional polyacrylamide gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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Most studies focused on physiological effects of exposure to various heavy metal ions and measuring heavy accumulation in different organisms, the effects of heavy metal toxicity at cellular level are still not adequately recognized. Various biomarkers are developed to indicate heavy metal toxicity in organisms, but most mechanisms of these biomarkers are not understood (Ling et al., 2009).

In order to study damage to the nervous system caused by heavy metals, the cerebral ganglion of Notarcus *leachii cirrosus* Stimpson (NLCS) was chosen as the experimental material. The marine aplysias have been workhorse for studying the neuronal circuits underlying specific behaviors. The aplysiid nervous system contains approximately 20,000 neurons which are organized into nine different ganglia. The neurons are very large and can be easily identified based on their sizes, electrical properties, and position in the ganglia (Akhmedov et al., 2014). In addition, the cerebral ganglia control many sense organs, such as antennas, oral cavity, and eyes. It is also related to motion and balance.

Heat shock protein families are common stress proteins and have homologous structures between species. High expression of Hsp 70 was detected under heavy metal stress (Ling et al., 2009). Thus these proteins can be used as biomarkers to monitor accumulation of heavy metals.

In the present work, the toxic effects of Cd and Pb on the aplysiid cerebral ganglion were evaluated by a combined approach involving ICP-MS, transmission electron microscopy, two dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and database search. Our study focused on the ultrastructure and protein expression differences in aplysia cerebral ganglia after treatment with Cd and Pb. In addition, we were interested in the correlation between these differently expressed proteins and developing efficient biomarkers to monitor heavy metals accumulation.

2. Materials and methods

2.1. Chemicals and animals

Analytical grade $CdCl_2$ and $PbCl_2$ were used in our experiments. NLCS were purchased from a merchant in Xiamen City, China, and were held in stock tanks filled with seawater at a constant temperature of $20 \pm 2\,^{\circ}C$. The salinity of the water was approximately 3%, and the pH of the water was controlled to 8.0, and the dissolved oxygen content was approximately 8 mg/L. The sea water was filtered to remove solid particles and sterilized with ultraviolet radiation before use. After acclimation to the environment for one week, the aplysias were divided into three groups. Two groups were exposed for 24 h to seawater containing 20 ppm $CdCl_2$ (NLCS-Cd) or $PbCl_2$ (NLCS-Pb), and the third group remained in seawater in the absence of heavy metal ions as the control. After treatment, cerebral ganglia were obtained from both the experimental and control groups using normal anatomical methods. Under the restrictions of season and region, aplysiid were very hard to obtain. According to the results from other fellows and our preliminary experiments, we found out that 20 ppm is the lethal concentration. Lethal duration is about 24–36 h (Chen et al., 2006). So we selected 20 ppm and 24 h which best effected on cerebral ganglion in aplysiid and did not lead to death of them.

2.2. Observation of the tissue with transmission electron microscopy

The cerebral ganglia were chopped into 1 mm^3 and pre-immobilized in 2.5% glutaraldehyde for 2 h. They were then washed with PBS buffer and immobilized with osmic acid solution (pH 7.4) at 4 °C. After immobilization, the samples were dehydrated with ethanol, embedded with epoxy resin and then sectioned. The ultrathin sections were dyed with uranyl acetate and lead citrate, and the dyed sections were observed with transmission electron microscopy under 120 kV.

2.3. Determination of Cd and Pb content using ICP-MS

The cerebral ganglia from the NLCS, NLCS-Cd and NLCS-Pb groups were quickly transferred into 1.5 mL centrifuge tubes at 4 °C, digested and their Cd and Pb contents determined using ICP-MS. Each measurement was carried out on three experimental replicates. Data were analyzed using Statistical Package for Social Sciences (SPSS) 16.0. Significant differences between treatment groups and control groups were tested by one-way analysis of variance (ANOVA).

2.4. Protein sample preparation

Fifteen cerebral ganglia from one group were obtained and homogenized at 4 °C in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 10 mM Tris, 1 mM EDTA, 0.5% ampholyte 3–10, 1% protease inhibitor cocktail). Then samples were held at 4 °C overnight for protein release and centrifuged at 100,000*g* for 15 min to remove the cell debris. The remaining supernatants were collected to obtain the proteome (Huang et al., 2016). Total protein content was measured using the Bradford assay (Bradford, 1976). All samples were kept at −80 °C before use.

2.5. Two dimensional gel electrophoresis (2D-GE)

2D-GE was performed as reported previously (Huang and Huang, 2012). Briefly, 150 μg protein sample was loaded onto 13 cm strips. Then isoelectric focusing (IEF) was performed using the carrier ampholyte (pH 5.0–8.0). The whole process for protein separation was carried out using an accumulative total of 10,000 Vh. Once the IEF was finished, it took 15 min to equilibrate the first dimension strip (equilibrium reagent, 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 1% DTT). Then it took 30 min to perform the SDS-PAGE (T = 12%) as the second dimension at 10 mA per gel, then at 25 mA per gel until the bromophenol blue reached the bottom edge of the gel. Three experimental replicates were performed for each sample (control, NLCS-Cd and NLCS-Pb).

2.6. Silver-staining and image analysis

Proteins on the gels could be observed using the silver-staining as described previously (Shen et al., 2007). The gels which were stained could be scanned with an Image Scanner II apparatus (GE Healthcare, Italy). Then the digitized images were analyzed using Image Master 2D Platinum software (Version 5.0, GE Healthcare, Italy). Protein spots were detected and matched between control and experimental groups. Then the individual spot volume values were acquired following the program instructions. In order to eliminate gel-to-gel diversity, every spot volume of each gel was normalized relative to the total valid spot volume. Afterwards, the proteins expressed with statistical differences were selected to be identified.

2.7. In-gel digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

Protein spots were excised manually and accurately from the 2D gels and washed with water. After destaining, the proteins