Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Differential protein expression of hippocampal cells associated with heavy metals (Pb, As, and MeHg) neurotoxicity: Deepening into the molecular mechanism of neurodegenerative diseases

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ARTICLE INFO

Keywords:
Metal exposure
Proteomics
Pathway visualization
Neurodegenerative diseases

ABSTRACT

Chronic exposure to heavy metals such as Pb, As, and MeHg can be associated with an increased risk of developing neurodegenerative diseases. Our in vitro bioassays results showed the potency of heavy metals in the order of Pb < As < MeHg on hippocampal cells. The main objective of this study was combining in vitro label free proteomics and systems biology approach for elucidating patterns of biological response, discovering underlying mechanisms of Pb, As, and MeHg toxicity in hippocampal cells. The omics data was refined by using different filters and normalization and multilevel analysis tools were employed to explore the data visualization. The functional and pathway visualization was performed by using Gene ontology and Pathvisio tools. Using these all integrated approaches, we identified significant proteins across treatments within the mitochondrial dysfunction, oxidative stress, ubiquitin proteome dysfunction, and mRNA splicing related to neurodegenerative diseases. The systems biology analysis revealed significant alterations in proteins implicated in Parkinson's disease (PD) and Alzheimer's disease (AD). The current proteomics analysis of three metals support the insight into the proteins involved in neurodegeneration and the altered proteins can be useful for metal-specific biomarkers of exposure and its adverse effects.

Significance: The proteomics techniques have been claimed to be more sensitive than the conventional toxicological assays, facilitating the measurement of responses to heavy metals (Pb, As, and MeHg) exposure before obvious harm has occurred demonstrating their predictive value. Also, proteomics allows for the comparison of responses between Pb, As, and MeHg metals, permitting the evaluation of potency differences hippocampal cells of the brain. Hereby, the molecular information provided by pathway and gene functional analysis can be used to develop a more thorough understanding of each metal mechanism at the protein level for different neurological adverse outcomes (e.g. Parkinson's disease, Alzheimer's diseases). Efforts are put into developing proteomics based toxicity testing methods using in vitro models for improving human risk assessment. Some of the key proteins identified can also potentially be used as biomarkers in epidemiologic studies. These heavy metal response patterns shed new light on the mechanisms of mRNA splicing, ubiquitin pathway role in neurodegeneration, and can be useful for the development of molecular biomarkers of heavy metals exposure.

Abbreviation: AD, Alzheimer's disease; APP, Amyloid precursor protein; Aβ, Amyloid-beta; CAM, Calmodulin; ETC, Electron transport chain; GAD, glutamate decarboxylase; Glu, Glutamate; GO, Gene ontology; LFQ, Label free quantification; LTP, Long term potentiation; MAPK, Mitogen-activated protein kinase; MAO-A, Monoamine oxidase A; NMDA, *N*-methyl D-aspartate; OD, Optical density; ORA, over-representation analysis; PD, Parkinson's disease; PS, Phosphatidyl serine; PI, Propidium iodide; PKC, protein kinase-C; ROS, Reactive oxygen species; UPS, Ubiquitin proteasome System

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https://doi.org/10.1016/j.jprot.2018.06.020
Received 20 March 2018; Received in revised form 26 June 2018; Accepted 26 June 2018

1. Introduction

Many environmental pollutants have been associated with human diseases [1]. Among various pollutants, heavy metals contribute to a great proportion of air, soil and water pollution and cause major health problems to human beings [2–4]. An increased worldwide industrialisation [5] has led to higher levels of pollution by potent neurotoxins such as lead (Pb), arsenic (As), and methyl mercury (MeHg) [6]. Both environmental and occupational exposures to any of the three metals are of significant toxicological concern [7, 8]. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide spreading in the environment [9, 10]. Heavy metal exposure can occur through contaminated air, food, water, and/or hazardous occupations [11–14]. The toxicity of heavy metals depends on several factors including dosage, route of exposure and chemical species, as well as on the age, gender, genetics, and nutritional status of exposed individuals [15–17]. Human exposure to three heavy metals (Pb, As, and MeHg) can disrupt brain function [18, 19], and increase the risk of diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [20–22].

These three heavy metals (Pb, As, and MeHg) are well known potent toxicants, and humans are exposed to each one of them via different routes [23, 24]. Firstly, the main routes of exposure for Pb are inhalation and ingestion [25, 26]. Inhalation exposure to Pb is a much more efficient route of absorption than ingestion [27, 28]. Pb metal is relatively common in the environment, and its toxicity impacts the children health [29]. Pb exposure in the workplace is responsible for a wide range of adverse effects, mainly on the brain [30]. Secondly, humans are exposed to various forms of As mainly via oral consumption of contaminated water, food or drugs [31, 32]. As metal can also enter the body via inhalation, which is particularly important for certain types of occupational exposure [33–35]. Also As metal is rapidly absorbed, distributed and stored in different body organs such as liver, kidney, and lung. Moreover, it can easily cross the blood brain barrier (BBB) and accumulate in the brain [36, 37]. Reported studies state that As metal has been directly linked to neurodegenerative diseases [38]. These findings raised concern over As induced neurotoxicity in humans [39–41]. However, the underlying molecular mechanisms not clear. Finally, MeHg contamination is possible through consumption of fish and other seafood [42, 43]. In humans, MeHg accumulates in kidneys and neurological tissues [44]. It is well known that ingested MeHg can interact with proteins due to its strong affinity to sulphur (-SH) containing functional groups, and cause organ dysfunction in the central nervous system [45, 46].

In the past few years, many studies have been conducted to understand the mechanisms underlying Pb, As and MeHg toxicity on the hippocampus region of the brain [47–49] Exposure to Pb and MeHg have significant effects on the human brain [50, 51]. Many reported evidence linked As exposure with developmental neurotoxicity [52]. Recently, Karri et al. reported that Pb, As, and MeHg exposure induce damage to the hippocampus region of the brain [53]. However, the risk level depends on exposure intensity and metal nature in the brain [39, 54]. Basha et al. observed that developmental exposure to Pb exhibits latent effects [20], through the epigenetic interaction of Pb with amyloid precursor protein (APP) gene causing neurodegeneration at an older age. As can disrupt the cognitive function of the brain in children [52]. MeHg directly disrupts the mitochondrial function by generating an uncontrolled release of Ca^{+2} [55], resulting in the dysregulation of the mitochondrial electron transport chain (ETC) [56] and causes the cell death. MeHg effect on mitochondria could be a potential cause of neurodegenerative diseases [57].

Most of the reported studies focused on the effects of metals on the brain in a generalized manner [46, 52, 58]. In the past few years, substantial improvements in toxicogenomics knowledge have led to an increase in the application of proteomics and systems biology knowledge to answer these mechanistic biological questions [59, 60]. To assess the biological effects of heavy metals, we are conducting studies engaging a systems toxicology approach that combines evaluation of classical toxicological endpoints with extensive molecular measurements and pathway analysis approaches [61–64]. The main goal of this project was taking advantage of high throughput proteomic technologies and systems biology tools for assessing the neurotoxicity mechanism of Pb, As, and MeHg on the hippocampal cells. We choose the mouse hippocampal HT-22 cell line due to the known sensitivity of chronic Pb, As, and MeHg exposure and relevant to the disease [65]. Here, a label-free quantitative proteomics approach [66] was used to detect the effects of Pb, As, and MeHg exposure at the protein level by using a non-cytotoxic dose (IC_{10}) of each metal to avoid secondary cytotoxic responses. The differential protein expression patterns involved in the heavy metal toxic response were quantified to deeply reveal the integrative molecular network of protein response to heavy metal stress. The proteomics data were integrated with a systems biology approach [67], in which the analysis tools Pathvisio, GO-elite, and Cytoscape were used to provide a more global view of the molecular] changes elicited by heavy metals in hippocampal cells [68, 69].

2. Materials and methods

2.1. Chemicals

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), and proteomics reagents: Urea (GE HealthCare, Life Sciences, CAS Number: 57-13-6), Sodium Dodecyl Sulphate (SDS) (Merck, CAS Number: 151-21-3), Ammonium Hydroxide (Fluka, CAS Number: 1066-33-7), Dithiothreitol (GE HealthCare, Life Sciences, CAS Number: 3483-12-3), Iodoacetamide (GE HealthCare, Life Sciences, CAS Number: 144-48-9), Formic Acid (Merck, CAS Number: 64-18-6), Acetonitrile (HPLC grade) (Fisher Chemical, CAS Number: 75-05-8), and Water (HPLC grade) (Fisher Chemical, CAS Number: 7732-18-5) are analytical grade and purchased from Sigma-Aldrich Química, S.L-Madrid (Spain).

2.2. Cell line and reagents

The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies [70]. The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]), 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37 °C. For all the experiments cells were grown at 70–80% confluence. The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.56×10^6 cells/mL and grown for 24 h (hrs.) before the metal treatment. Duplicates wells of cells were treated with 10 exposure levels of Pb, As, and MeHg ranging from 10 to 100 µM, 0.4 to 4.2 µM, and 0.6 to 12 µM, respectively; due to the 8 days exposure-period, medium containing the given concentration was refreshed at 2 days interval in order to maintain the metal exposure along time. Metal stock solutions 100× was prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO was added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution for each individual metal were prepared by prior dilution of the stock solution in phosphate buffered saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Cytotoxicity

The MTT assay was carried out using a modification of the method