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Methylmercury exposure alters RNA splicing in human neuroblastoma SK-N-SH cells: Implications from proteomic and post-transcriptional responses☆

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ABSTRACT

The neurotoxic effects of methylmercury (MeHg) have been intensively studied. However, the molecular mechanisms responsible for the neurotoxicity of MeHg are not fully understood. To decipher these mechanisms, proteomic and high-throughput mRNA sequencing (RNA-seq) technique were utilized, comprehensively evaluating the cellular responses of human neuroblastoma SK-N-SH cells to MeHg exposure. Proteomic results revealed that MeHg exposure interfered with RNA splicing via splicesome, along with the known molecular mechanisms of mercury-related neurotoxicity (e.g. oxidative stress, protein folding, immune system processes, and cytoskeletal organization). The effects of MeHg on RNA splicing were further verified using RNA-seq. Compared to control, a total of 658 aberrant RNA alternative splicing (AS) events were observed after MeHg exposure. Proteomics and RNA-seq results also demonstrated that mercury chloride ($HgCl_2$) influenced the expression levels of several RNA splicing related proteins and 676 AS events compared to control. These results suggested that RNA splicing could be a new molecular mechanism involved in MeHg and $HgCl_2$ neurotoxicity.

1. Introduction

Methylmercury (MeHg) is among the most well-known and toxic organometals (Agusa et al., 2005; Marques et al., 2014b; Skinner et al., 2007). Particularly toxic to the central nervous system (CNS) (Carocci et al., 2014), the lipophilic MeHg can easily cross blood-brain barrier (Golding et al., 2013) and induce neurotoxicity (Kampa and Castanas, 2008). Cellular responses to MeHg and related neurotoxic effects have been extensively studied over the past few decades. It has been found that MeHg can disrupt cellular redox homeostasis through the generation of reactive oxygen species (ROS) (Aschner et al., 2007). In fact, ROS production is generally considered as a crucial event in mediating the neurotoxicity of low micromolar concentrations of MeHg. This has been validated in several cell models, including neurons, astrocytes, neuroblastomas, and microglial cells (Ni et al., 2010; Sanfeliu et al., 2001). Furthermore, MeHg-induced ROS generation has been shown to alter brain functions, resulting in pathophysiological injury (Huang et al., 2011). At low micromolar concentrations, MeHg can also disrupt calcium homeostasis in various cell types (e.g. neurons, neuroblastoma cells, and Purkinje cells) (Castoldi et al., 2001). Cytoskeletal components, especially microtubules, appear to be crucial targets for MeHg (Castoldi et al., 2001). Indeed, MeHg has been shown to promote microtubule disruption and further neuronal network dissolution *in vitro* (Castoldi et al., 2001). The linkage between mercury exposure and neurodegenerative diseases was also verified using animal models (Fujimura et al., 2009; Pendergrass et al., 1997). Corroborating these results, epidemiologic studies suggested that mercury contributed to the etiology of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (Gorell et al., 1999; Marques et al.,

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2014a; Mutter et al., 2004).

The toxicological effects of MeHg in neural cells have been largely investigated using traditional biochemical approaches. These studies generally focused on a limited number of molecular targets or mechanisms of action. Although several putative mechanisms were characterized, the neurotoxicity of MeHg has yet to be fully elucidated. Omics-based techniques (transcriptomics and proteomics) can enable the simultaneous detection of numerous changes of biological functions. The joint analysis of transcriptome and proteome were successfully used for analysis of regulatory response of cells/tissues. However, to the best of our knowledge, no-related combined omics-based approaches were used to investigate mercury neurotoxicity. Several proteomic-based approaches were used to characterize MeHg neurotoxicity. Amongst the published proteomic studies, most focused on analyzing protein expression profile changes in the tissues of fish or rat brains (Keyvanshokooh et al., 2009; Kong et al., 2013; Wang et al., 2011). Unfortunately, just as traditional biochemical approaches, only some similar mechanisms (e.g. mitochondrial dysfunction, oxidative stress, altered calcium homeostasis, energy metabolism, and protein dysfunction) were found in these proteomic studies. Some studies using rat/mice neural cells have been conducted, however, the obtained results were not sufficiently explored (Vendrell et al., 2007, 2010). Furthermore, due to the inter-specific variations, these toxicity researches based on animals-related cell models or experimental animals were not always applicable to human beings. It is necessary to conduct toxicity tests based on human biology relevant models. However, proteomics-related work on MeHg neurotoxicity using neural cells from human source has yet to be reported.

Compared to MeHg, the neurotoxic effects of inorganic mercury (I-Hg) on neural cells and animal brains are much less known. Indeed, much less scientific effort has been devoted to the characterization of I-Hg neurotoxicity. This is primarily because I-Hg is lipophobic and thus considered to have limited capacity to cross the blood-brain barrier. However, I-Hg could be of neurotoxic concern based on the following reasons. First, it has been shown that MeHg can be demethylated to the inorganic form (Hg^{2+}) in the brain (Vahter et al., 1995). In fact, I-Hg has actually been detected within the CNS in humans and *Macaca fascicularis* monkeys following chronic MeHg exposure (Vahter et al., 1995). Second, I-Hg may accumulate in the brains of young organisms with incompletely developed blood-brain barrier (Patrick, 2002). Third, it has been shown that mercury can accumulate in the adult fish and rat brains in response to I-Hg exposure. Actually, high concentrations of mercury have been detected in the brains of mercury chloride ($HgCl_2$) exposed rats and zebrafish (Gonzalez et al., 2005; Magos et al., 1985). Fourth, chronic exposure of I-Hg has been shown to cause CNS damage in human beings (Holmes et al., 2009). Finally, I-Hg can be more potent than MeHg in terms of cellular function disruption (e.g. inhibition of glutamine synthetase activity in cultured astrocytes) (Allen et al., 2001). Given the above information, the modes of actions of I-Hg in neural cells and brains deserve further study.

Currently, the neurotoxic effects of MeHg on human relevant neural cells are not fully understood, especially concerning proteomic mechanisms of action. To our knowledge, all proteomics studies investigating the neurotoxicity of mercury focused on only one kind of mercury species at a time. Human beings are exposed to mercury compounds through a long chronic low-dose exposure process. This relative exposure dose has no obvious adverse health effect/lethal effect to human beings. In this study, the neurotoxicity of MeHg at concentration with no obvious effect on the cell viability of SK-N-SH cells was investigated using proteomic-based methods. The toxicity effects of $HgCl_2$ were also investigated. Changes in the proteomic profiles of SK-N-SH cells were investigated using two-dimensional electrophoresis (2-DE). Bioinformatical analysis was conducted to characterize the molecular mechanisms of MeHg neurotoxicity. As RNA splicing was found to be a novel molecular mechanism of MeHg-mediated neurotoxicity in the SK-N-SH cells, high-throughput mRNA sequencing (RNA-seq) was further conducted to confirm the effects of MeHg on RNA splicing. The present investigation provides valuable information on not only the proteomic profiles of SK-N-SH cells in responsible to MeHg and $HgCl_2$, but also the influences of these two mercury compounds to RNA splicing, which was not reported before.

2. Materials and methods

2.1. Materials

3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), dithiothreitol (DTT), iodoacetamide (IAA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Phosphate Buffered Saline (DPBS) and penicillin/streptomycin were bought from HyClone (Logan, UT, USA). High glucose ($4.5\ g/L^{-1}$) Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). TRIzol was supplied by Life Technologies (Gaithersburg, MD, USA). $HgCl_2$ ($\geq$99.5%) was obtained from Beijing Chemical Reagents Company (Beijing, China). The stock solution of $HgCl_2$ was made using sterile distilled water. The stock solution of MeHg was prepared by dissolving MeHgCl (Merck, Darmstadt, Germany) in DMSO. Exposure solutions were made via dilution of stock solutions with cell culture medium. Final DMSO concentrations were less than 0.1%. All chemicals used were of analytical grade or higher.

2.2. Cell line and cell viability assay

The human neuroblastoma cell line SK-N-SH was purchased from the Cell Resource Center of the Institute of Basic Medical Sciences (Beijing, China). Cells were cultured in DMEM supplemented with 10% FBS and 1% 100 units/ml penicillin/streptomycin. The cells were grown in an incubator at 37 °C with 5% volatile CO_2.

SK-N-SH cells were seeded in 96-well plates at a density of 3×10^4 cells per well and grown to the desired cell confluency (70–80%). The medium was then removed and replaced with 200 μL of new medium containing a range of MeHg (0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 μM) or $HgCl_2$ (1, 5, 10, 50, 100, 500, and 1000 μM) concentrations. After 72 h, 100 μL of medium was removed and 10 μL of Alamar Blue (100 μM) was added into the wells. Plates were then incubated for 1 h at 37 °C in the dark. Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength using a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). The experiment was performed independently two times. Six parallel wells ($n = 6$) were designed for each exposure condition during every test.

2.3. Protein preparation for two-dimensional electrophoresis

After MeHg or $HgCl_2$ treatment, cells were washed twice with pre-chilled DPBS and lysed using 700 μL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, protease inhibitor cocktail, and nuclease) per 100 cm^2 cell culture dish. Samples were then incubated on ice for 1 h. Protein was collected by centrifugation (42 000 rpm, 40 min, 4 °C). The supernatant was precipitated with 4 vol of acetone (overnight, −20 °C) and centrifuged at 12000 rpm for 20 min at 4 °C. Samples were resuspended with 2 M thiourea,