NeuroToxicology 59 (2017) 197–209

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Methylmercury-induced developmental toxicity is associated with oxidative stress and cofilin phosphorylation. Cellular and human studies

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 13 May 2016
Accepted 27 May 2016
Available online 27 May 2016

Keywords:
Methylmercury
Cofilin
Actin
Mitochondria
Oxidative stress
Cultured neurons
Human placenta

ABSTRACT

Environmental exposure to methylmercury (MeHg) during development is of concern because it is easily incorporated in children's body both pre- and post-natal, it acts at several levels of neural pathways (mitochondria, cytoskeleton, neurotransmission) and it causes behavioral impairment in child. We evaluated the effects of prolonged exposure to 10–600 nM MeHg on primary cultures of mouse cortical (CCN) and of cerebellar granule cells (CGC) during their differentiation period. In addition, it was studied if prenatal MeHg exposure correlated with altered antioxidant defenses and cofilin phosphorylation in human placentas (n = 12) from the INMA cohort (Spain).

Exposure to MeHg for 9 days in vitro (DIV) resulted in protein carbonylation and in cell death at concentrations ≥200 nM and ≥300 nM, respectively. Exposure of CCN and CGC to non-cytotoxic MeHg concentrations for 5 DIV induced an early concentration-dependent decrease in cofilin phosphorylation. Furthermore, in both cell types actin was translocated from the cytosol to the mitochondria whereas cofilin translocation was found only in CGC. Translocation of cofilin and actin to mitochondria in CGC occurred from 30 nM MeHg onwards. We also found an increased expression of cortactin and LIMK1 mRNA in CGC but not in CCN. All these effects were prevented by the antioxidant probucol.

Cofilin phosphorylation was significantly decreased and a trend for decreased activity of glutathione reductase and glutathione peroxidase was found in the fetal side of human placental samples from the highest (20–40 μg/L) MeHg-exposed group when compared with the low (<7 μg/L) MeHg-exposed group.

In summary, cofilin dephosphorylation and oxidative stress are hallmarks of MeHg exposure in both experimental and human systems.

1. Introduction

Methylmercury (MeHg), a relevant persistent environmental contaminant, is widely recognized as a potent neurotoxicant in humans (WHO, 2007) since it may affect both the developing and the mature central nervous systems (CNS). Ingestion of contaminated fish is the primary source of MeHg exposure (Mergler et al., 2007; Ramon et al., 2011; Vieira et al., 2015; Xu and Newman, 2015) and both developmental and aging states are thought to exacerbate the neurotoxic effects of this organometal with primary signs of neurological dysfunction. In highly exposed populations

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http://dx.doi.org/10.1016/j.neuro.2016.05.018
0161-813X/

these signs include cerebellar ataxia, constriction of the visual fields and sensory disturbances as it was observed in patients affected by the Minamata disease (Ekino et al., 2007). The most susceptible cerebral regions to MeHg-mediated injury are the cerebellum, more specifically the cerebellar granule cells (CGC), the calcarine area and the postcentral gyrus whereas no-significant differences are found in the white matter of the corpus callosum (Korogi et al., 1994; Sanfeliu et al., 2003). At the intracellular level MeHg-induced cytotoxicity in neurons has been ascribed to three major mechanisms: perturbation of intracellular Ca^{+2} levels, generation of oxidative stress and interactions with critical sulphydryl groups (Castoldi et al., 2001; Do Nascimento et al., 2008). More recently, the interaction of MeHg with seleno amino acids has been reported (Farina et al., 2009; Khan and Wang, 2009). Hallmarks of MeHg neurotoxicity in experimental *in vivo* and *in vitro* models include apoptosis, inhibition of protein synthesis, microtubule disruption or depolymerization and disturbance of neurotransmission (Yoshino et al., 1966; Castoldi et al., 2000, 2001; Dare et al., 2000, 2001; Fonfría et al., 2001, 2005; Ceccatelli et al., 2010; Hogberg et al., 2010).

One of the main intracellular targets for MeHg neurotoxicity is the mitochondrion (Castoldi et al., 2001), where this organometal is early accumulated in the brain following its administration (Yoshino et al., 1966). This accumulation has been associated to changes in mitochondrial morphology in the developing rat brain (O'Kusky 1983) and loss of the mitochondrial membrane potential (Castoldi et al., 2000). Likewise, mitochondria and cytoskeletal alterations such as neurite degeneration and neuronal network fragmentation have been reported to occur preceding MeHg-induced apoptosis in cultured cerebral cortical neurons (CCN) (Fujimura et al., 2009) and CGC neurons (Castoldi et al., 2000; Vendrell et al., 2007, 2010). In this way, cofilin, a cytoskeletal protein member of the actin depolymerizing factor (ADF), was previously found in mitochondrial fractions after exposure to MeHg in cultured CGC (Vendrell et al., 2010). Cofilin is a low-molecular weight cytosolic protein ubiquitously expressed which is present in both phosphorylated (P-cofilin) and non-phosphorylated states (non-P-cofilin). This protein regulates actin dynamics by promoting the depolymerization and severing of actin filaments, and regulating the recycling of the resulting monomers (Gourlay and Ayscough, 2005). Thus, the balance between non-P-cofilin and P-cofilin facilitates actin filament turnover whose dynamics and reorganization are responsible for key events modulating neuron survival such as neuron shape and polarity, as well as dendrite spine physiology including spine formation and maintenance, receptor trafficking and synaptic plasticity (Spence and Soderling, 2015). There is also a redox regulation of cofilin via its four cysteine residues; in fact, cofilin oxidation has been reported in different cell types and under different oxidative conditions (reviewed by Samstag et al., 2013). Also, it has been suggested that cofilin has an important function during the initiation phase of apoptosis, being the mitochondrial translocation of cofilin an essential step for engaging the apoptotic pathway (Chua et al., 2003; Li et al., 2013). In this sense, Samstag et al. (2013) have identified that cofilin oxidation is the molecular switch that targets cofilin to the mitochondria in T-cells. Furthermore, mitochondrial association of cofilin during apoptosis is preceded by actin, which translocates to mitochondria during apoptotic cell death. On the other hand, dephosphorylated cofilin (Ser 3) is the cofilin form that is translocated to mitochondria (Li et al., 2015b). In previous works, we described that primary cultures of CGC showed a reduction of P-cofilin in response to MeHg exposure along with increased levels of non-P-cofilin in mitochondrial fractions (Vendrell et al., 2010).

Moreover, MeHg can lead to the generation of oxidative stress through interaction with selenol and thiol groups, as pointed out above. Therefore, it is probable that, in addition to bind to specific thiol-containing proteins, MeHg can also bind in a stable way to selenoproteins, such as antioxidant enzymes, glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) (Farina et al., 2011). These proteins are important components of the cellular antioxidant system, and their inhibition contributes to the disruption of the normal redox balance of brain cells. According to this, it has been proposed that antioxidants such as propyl gallate and probucol (PB) may be used as neuroprotective agents (Gassó et al., 2001). In fact, the beneficial effects of PB have been found to be correlated with increased GPx-1 activity and decreased lipid peroxidation (Farina et al., 2009).

In the present work, we have further investigated the intracellular changes in P-cofilin and cofilin/actin localization after long-term exposure to low MeHg concentrations by using an *in vitro* paradigm that compares two cell types differentially affected by MeHg: mice CGC and CCN. After confirming that MeHg induced cofilin dephosphorylation and oxidative stress in both cell types we studied these variables in human placentas from individuals with known MeHg exposure since this tissue mediates the transfer of mercury from mothers to fetuses during the gestational period.

2. Experimental procedures

2.1. Materials

Pregnant NMRI mice (16th day of gestation) and 7-day-old NMRI mice were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Dulbecco's modified Minimum Essential Medium (DMEM) was from Biochrom (Berlin, Germany). Rabbit polyclonal IgG anti-cofilin 1 and anti-Ser 3 P-cofilin 1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-β actin primary antibody was from Sigma (St. Louis, MO) and protein A/G-horseradish peroxidase-conjugated secondary antibody was from Jackson Laboratories (Baltimore, USA). Protein carbonyl enzyme immuno-assay kit (BIOCELL PC TEST) was from BioCell Corporation Ltd. (Auckland, NZ). MeHg was from ICN (Cleveland, Ohio, USA). Any others reagents including jasplakinolide (JAS) were also purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Human placentas belonged to participants from the INMA-Valencia mother-infant cohort (Spain) (Childhood and Environment; www.proyectoinma.org). Placenta samples were collected at delivery in 2004, and pieces of maternal and fetal sides were immediately dissected, coded, frozen and stored confidentially and anonymously at −80 °C until processed. A subset of 12 placental samples was selected as function of the total mercury concentrations measured in cord blood (low exposure (<7 μg/L) and high exposure (20–40 μg/L)). Informed consent was obtained from all participants and the study was approved by the Hospital La Fe Ethics Committee (Valencia, Spain).

2.2. Neuronal cell culture

Primary cultures were prepared as previously described (Farina et al., 2009; Vendrell et al., 2010; Regueiro et al., 2015). Both CGC and CCN cells were seeded (1.6×10^6 cells/mL, except otherwise stated) on multi-well-plates precoated with poly-D-lysine and incubated in a modified DMEM solution (31 mM glucose and 0.2 mM glutamine), supplemented with insulin, penicillin and 10% foetal calf serum, containing 5 mM KCl for CCN and 25 mM KCl for CGC in a humidified 5% CO2/95% air atmosphere at 37 °C without changing the culture medium, unless otherwise specified. A mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added to the cultures after 24 h incubation to prevent glial