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Sex- and structure-specific differences in antioxidant responses to methylmercury during early development

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ABSTRACT

Methylmercury (MeHg) is a ubiquitous environmental contaminant and neurotoxin, particularly hazardous to developing and young individuals. MeHg neurotoxicity during early development has been shown to be sex-dependent via disturbances in redox homeostasis, a key event mediating MeHg neurotoxicity. Therefore, we investigated if MeHg-induced changes in key systems of antioxidant defense are sex-dependent. C57BL/6J mice were exposed to MeHg during the gestational and lactational periods, modeling human prenatal and neonatal exposure routes. Dams were exposed to 5 ppm MeHg via drinking water from early gestational period until postnatal day 21 (PND21). On PND21 a pair of siblings (a female and a male) from multiple (5–6) litters were euthanized and tissue samples were taken for analysis. Cytoplasmic and nuclear extracts were isolated from fresh cerebrum and cerebellum and used to determine thioredoxin (Trx) and glutathione (GSH) levels, as well as thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) activities. The remaining tissue was used for mRNA analysis. MeHg-induced antioxidant response was not uniform for all the analyzed antioxidant molecules, and sexual dimorphism in response to MeHg treatment was evident for TrxR, Trx and GPx. The pattern of response, namely a decrease in males and an increase in females, may impart differential and sex-specific susceptibility to MeHg. GSH levels were unchanged in MeHg treated animals and irrespective of sex. Trx was reduced only in nuclear extracts from male cerebella, exemplifying a structure-specific response. Results from the gene expression analysis suggest posttranscriptional mechanism of sex-specific regulation of the antioxidant response upon MeHg treatment. The study demonstrates for the first time sex-and structure-specific changes in the response of the thioredoxin system to MeHg neurotoxicity and suggests that these differences in antioxidant responses might impart differential susceptibility to developmental MeHg exposure.

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1. Introduction

Methylmercury (MeHg) is an environmental pollutant that targets the central nervous system (CNS) and causes severe neurological deficits (Bisen-Hersha et al., 2014; Fischer et al., 2008; Manfroi et al., 2004; Sanfeliu et al., 2003). This is particularly true for newborn and young individuals, which are more susceptible to the toxin due to undeveloped blood-brain barrier (BBB) and lower excretion capacity (Fischer et al., 2008; Manfroi et al., 2004). Targeting the brain by MeHg during early periods of development, when critical processes, such as cell division and neuronal migration take place, leads to irreversible damage, as shown in numerous epidemiological (Llop et al., 2013) and experimental studies (Fischer et al., 2008; Gimenez-Llort et al., 2001; Manfroi et al., 2004). It is noteworthy that sexual dimorphism in response to developmental MeHg exposures has been reported, with males showing increased susceptibility to MeHg than females in behavioral evaluations (Björklund et al., 2007; Gimenez-Llort et al., 2001; Llop et al., 2013; Rossi et al., 1997). However, because of scarce biochemical data, the mechanisms underlying these differences have yet to be elucidated.

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Oxidative stress and disrupted antioxidant protection is a key event in MeHg neurotoxicity. It is believed to be a consequence of MeHg interacting with nucleophilic groups, especially sulfhydryl and selenohydryl groups present in numerous antioxidant molecules (Farina et al., 2011; Farina et al., 2013). The thioredoxin (Trx) and glutathione (GSH) systems are major antioxidant systems targeted by MeHg (Farina et al., 2011; Farina et al., 2013). The Trx system consists of two major isoforms of Trx and Trx-regenerating selenoproteins – thioredoxin reductases (TrxR); Trx1/TrxR1 is present in both cytoplasm and nuclei, and Trx2/TrxR2 is localized in mitochondria (Aon-Bertolino et al., 2011; Lu and Holmgren, 2014; Silva-Adaya et al., 2014). The Trx system is expressed in the CNS, with higher levels of Trx in neurons and TrxR in astrocytes (Lippoldt et al., 1995; Rozell et al., 1985; Rubartelli et al., 1992; Rybnikova et al., 2000; Silva-Adaya et al., 2014), indicating on important role of Trx in neuronal differentiation and regeneration (Endoh et al., 1993; Masutani et al., 2004). The system provides the reducing equivalents for thioredoxin-dependent peroxiredoxins (Prx), which can efficiently remove reactive oxygen species (ROS). Moreover, Trx system regulates the activities of numerous oxidative-sensitive molecules, such as ribonucleotide reductases, methionine sulfoxide reductases, transcription factors, caspases and kinases, and is involved in the repair of oxidized proteins (Lu and Holmgren, 2014; Silva-Adaya et al., 2014). The redox homeostasis is also maintained by system using glutathione (GSH), the most abundant low molecular thiol present in various cellular compartments (Conrad et al., 2013; Go and Jones, 2010). Oxidized glutathione (GSSG) is reduced back to its reduced form (GSH) by glutathione reductase (GR) and utilized by antioxidant enzymes, such as glutathione peroxidases (GPx) and glutaredoxins (Grx). It is noteworthy that both the GSH and Trx antioxidant systems act cooperatively in the homeostatic maintenance of the redox state in cells (Conrad et al., 2013; Go and Jones, 2010).

The antioxidant response to MeHg neurotoxicity has been studied in various experimental models, showing disruption of both the Trx and GSH systems (Farina et al., 2011; Farina et al., 2013). However, the preponderance of studies, conducted primarily in males, overlooks the fact that the redox system is differentially regulated in males *vs.* females (Benner et al., 2013; Borrás et al., 2003; Kenchappa et al., 2004; Malorni et al., 2007; Marotti et al., 2010). For example, sex hormones have been shown to affect both oxidative stress-promoting molecules and endogenous antioxidant, most likely through transcriptional and post-transcriptional regulation (Benner et al., 2013; Ejima et al., 1999; Kenchappa et al., 2004; Marotti et al., 2010). The same studies suggest more efficient antioxidant protection in females *vs.* males (Benner et al., 2013; Ejima et al., 1999; Kenchappa et al., 2004; Marotti et al., 2010). In fact, one study demonstrated positive effect of the female hormone, 17-β-estradiol, in suppressing MeHg-induced neurotoxicity in adult male mice (Malagutti et al., 2009). These observations led us to hypothesize that the relative resistance of young females to MeHg neurotoxicity might be secondary to differential regulation of their antioxidant systems. Since Trx was previously shown to be upregulated by female hormones (Ejima et al., 1999; Lee et al., 2003), we focused herein predominantly on the Trx system.

2. Materials and methods

2.1. MeHg treatment

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Adult, twelve-weeks-old C57BL/6J mice were maintained at standard conditions with free access to food and water. Eight groups, each containing one male and two females, were kept together for ten days. Next, pregnant females were separated and randomly assigned to the control or treatment groups (eight mice in each). The control group received drinking water and the treated group received water with 5 ppm methylmercury (II) chloride (MeHg) (Sigma-Aldrich, #442534), which produces exposure of about 400 μg/kg/day (Newland and Reile, 1999). Fresh MeHg solutions were replaced weekly, made from 50 ppm stock. Water bottles were not available for pups, so their exposure was only via maternal milk. Treatment was started immediately after dams' assignment into groups and carried out until postnatal day 21 (PND21). Next, the pups were sacrificed by decapitation preceded with isoflurane anesthesia. Samples from cerebrum and cerebellum were isolated on wet ice and used for extraction or frozen at −80 °C. Samples of siblings, female and male from multiple litters, were used for the analysis. All the pups from two of the control group and one of the treated group died. Moreover, same-sex siblings were inherent to three litters; therefore, the final number of analyzed samples was 5–6: 5 males and 6 females from control litters and 6 males and 6 females from treated litters. No sex-specific effect was observed in pups' survival, at PND21 an average% of male pups in litter was 48,19 (±26,98) for control and 51,69 (±32,79) for MeHg-treated group.

2.2. Nuclear and cytoplasmic extracts isolation

Nuclear and cytoplasmic extracts were isolated from 80 mg of fresh tissues by gentle lysis and rapid centrifugation with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, #78835), according to the manufacturers' protocol, and frozen at −80 °C for further analysis.

2.3. Biochemical determinations

TrxR activity was measured using a Kit for Assay of Mammalian Thioredoxin Reductase (Cayman –IMCO Corp., #FkTRXR-03), according to the manufacturer's protocol. Approximately 10 μg of protein extract was used in a reaction mixture. The results were compared with standard curve for active TrxR (in nM) and presented as nmol/mg of total protein.

Reduced Trx level was measured using a Kit for Assay of Thioredoxin (Cayman –IMCO Corp., #FkTRX-02-V2), according to the manufacturer's protocol. Approximately10 μg of protein extract was used in a reaction mixture. The results were compared with standard curve for active Trx (in ng) and presented as μg/mg of total protein.

GPx activity was measured using a Glutathione Peroxidase Assay Kit (Cayman, #703102), according to the manufacturer's protocol. Approximately 50 μg of protein extract was used in a reaction mixture. The results were calculated as nmol/(min · mg of total protein).

Total glutathione (GSH) level was measured using method based on enzymatic recycling reaction (Baker et al., 1990), performed as described previously in (Caito and Aschner, 2015). Diluted 5x extract was used in a reaction mixture. The results were compared with standard curve for reduced GSH and calculated as nmol/mg of total protein.

All results were standardized to total protein content, determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, #23225), according to the manufacturer's protocol.

2.4. Gene expression assay

Gene expression was evaluated with quantitative real time PCR method (qRT- PCR). Total RNA was isolated from 50 mg of frozen tissue using the standard protocol for Trizol Reagent (Ambion, #10296028) as was described previously (Livak and Schmittgen,

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