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Methylmercury interferes with glucocorticoid receptor: Potential role in the mediation of developmental neurotoxicity

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ABSTRACT

Methylmercury (MeHg) is a widespread environmental contaminant with established developmental neurotoxic effects. Computational models have identified glucocorticoid receptor (GR) signaling to be a key mediator behind the birth defects induced by Hg, but the mechanisms were not elucidated. Using molecular dynamics simulations, we found that MeHg can bind to the GR protein at Cys736 (located close to the ligand binding site) and distort the conformation of the ligand binging site. To assess the functional consequences of MeHg interaction with GR, we used a human cell line expressing a luciferase reporter system (HeLa AZ-GR). We found that 100 nM MeHg does not have any significant effect on GR activity alone, but the transactivation of gene expression by GR upon Dex (a synthetic GR agonist) administration was reduced in cells pre-treated with MeHg. Similar effects were found in transgenic zebrafish larvae expressing a GR reporter system (SR4G). Next we asked whether the effects of developmental exposure to MeHg are mediated by the effects on GR. Using a mutant zebrafish line carrying a loss-of-function mutation in the GR (gr^{S357}) we could show that the effects of developmental exposure to 2.5 nM MeHg are mitigated in absence of functional GR signaling. Taken together, our data indicate that inhibition of GR signaling may have a role in the developmental neurotoxic effects of MeHg.

1. Introduction

Methylmercury (MeHg) is a widespread environmental contaminant produced by microbial activity that converts inorganic mercury released in the environment by natural as well as anthropogenic sources (Driscoll et al., 2013). It has high affinity for sulfur-containing anions, particularly the thiol (-SH) groups on the amino acid cysteine (Kerper et al., 1992). The methylmercuric-cysteinyl complex is recognized as methionine by amino acid transporters, and is thus transported freely throughout the body including across the blood-brain barrier and the placenta (Kerper et al., 1992). Epidemiological and experimental studies have shown that MeHg is a potent neurotoxicant particularly durig development (for review, see (Castoldi et al., 2008; Johansson et al., 2007)). There is also evidence that in humans and aquatic animals MeHg affects various endocrine axes, including the hypothalamic-pituitary-adrenal (HPA) axis (see (Tan et al., 2009; Waye and Trudeau, 2011)). The intracellular events leading to these alterations are not elucidated, but may include oxidative stress, changes in intracellular calcium homeostasis, interference with sulfhydryl groups, which are known effects induced by MeHg (Ceccatelli et al., 2013). More recently, the glucocorticoid (GC) receptor (GR) signaling pathway has been identified as a possible target for heavy metals, such as arsenic and Hg, linking metal exposure with developmental disorders (Ahir et al., 2013).

GC signaling plays a critical role during organogenesis, inducing the maturation of several organs (Harris and Seckl, 2011; Khulan and Drake, 2012). In particular GCs promote brain development by initiating terminal maturation, remodeling of axons and dendrites, and regulating cell survival (Cameron and Gould, 1994; Meyer, 1983; Yehuda et al., 1989). GCs exert their biological action by binding to, and activating the GR – a multidomain ligand-activated nuclear receptor. Upon agonist binding, GR is released from the chaperones and translocates into the nucleus as homodimer (Müller et al., 2002; Oasa et al., 2015). GR can regulate gene expression in two ways: (1) by interaction with specific DNA sequences, defined as GC-responsive elements (GRE), located in promoter regions; or (2) without directly

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binding DNA, but by interacting with other DNA-bound proteins or other molecules involved in intracellular signaling (Frego and Davidson, 2006; Schoch et al., 2010; Yudt et al., 2003; Beck et al., 2009). The functionality of GR is linked to the position of the terminal α helix 12 (H12) (Veleiro et al., 2010), and the structural stability of the neighbouring H11 (Yudt et al., 2003).

In light of the fact that GC signaling modulates the expression of several genes that may have a role in neurodevelopment and/or predispose to neurodevelopmental disorders (Bose et al., 2010, 2015), we investigated whether MeHg interferes with the GR using a combination of *in silico*, *in vitro* and *in vivo* assays with the final aim of linking a possible MeHg/GR interaction to MeHg developmental neurotoxic effects.

2. Materials and methods

2.1. Molecular dynamics simulations

2.1.1. System preparation

We have selected the synthetic GC analog dexamethasone (Dex) as GR agonist for molecular dynamics (MD) simulations, and prepared the following systems: GR-Dex (GR holo), GR-Dex-free (GR apo), and two additional systems with mercurated Cys residue at position 736 (Cys736), one for holo and the other for the apo form. The Cys736 residue, located on H11, was selected as potential target for the binding of MeHg due to its position in the immediate vicinity of the ligand-binding pocket, as well as due to its proximity to other residues directly participating to ligand binding. The models were prepared based on the chain A monomer of the X-ray crystal structure of the GR-Dex complex (PDB entry 1M2Z (Bledsoe et al., 2002)) from *R. norvegicus*, which has a 100% sequence identity with the GR ligand binding domain (LBD) in *H. sapiens*. All mutations introduced into the GR protein for the purpose of crystallisation were reverted to the wild type. The apo form was prepared by the removal of the coordinates for the Dex ligand. The models were pre-processed in Maestro (Schrödinger Release, 2014) using OPLS_2005 force field (Hornak et al., 2006; Kaminski et al., 2001) with the protonation states assigned by PropKa (Li et al., 2005) at pH 7.5. The protonation states were then inspected manually on the basis of the hydrogen bonding networks deduced from the X-ray structure. The histidine residues were assigned a neutral charge with the protonation of the imidazole ring at Nδ in H654, while Nε was protonated in H588, H726, and H775.

Each model was solvated with the 15,860 TIP3P (Jorgensen et al., 1983) waters in an 82.0 Å × 82.0 Å × 82.0 Å water box, adding 45 Na^+ and 44 Cl^- ions to neutralise the systems. All Na^+ and Cl^- ion were placed randomly within the water box using the Monte Carlo sampling procedure provided by CHARMM-GUI (Jo et al., 2008; Lee et al., 2016). The protein was described by the all-atom additive CHARMM36 (MacKerell et al., 1998) force field. CMAP (MacKerell et al., 2004) correction was applied to the ϕ and ψ angles in the protein backbone to improve its dynamic and structural properties. For the Dex ligand, the parameters produced by CGenFF (Vanommeslaeghe et al., 2010) were adopted without additional optimization. For the MeHg moiety, the parameters were determined by the RESP (Bayly et al., 1993) algorithm available in the Antechamber (Wang et al., 2006) program in the gas phase at the B3LYP (Becke, 1993; Stephens et al., 1994) and MP2 (Head-Gordon et al., 1988) level of calculations. The van der Waals radii for the mercury (σ_{Hg} = 1.0 Å) and sulfur (σ_S = 2.0 Å) atoms and the corresponding Lennard-Jones well depths (ε_{Hg} = −1.0 kcal/mol; ε_S = −0.45 kcal/mol) were based on Fuchs et al. (2006).

2.1.2. Molecular dynamics simulation

Each prepared system was first energy minimized by (steepest descent) gradient optimizations until the maximum force acting on each atom of 1000 kJ/mol nm and the minimization was carried out using LINCS (Hess, 2008). The systems were then equilibrated with position restraints to avoid large structural distortion of the minimized protein during the equilibration. First, under the constant volume and temperature (NVT) conditions, the systems were heated to 298.15 K and equilibrated for 25 ns with the temperature controlled by the extended temperature Nosé-Hoover thermostat (Hoover, 1985). In the subsequent 1 ns equilibration, the constant pressure and temperature (NPT) conditions were applied for the equilibration of the system volume at 1 bar. The pressure was controlled using the Berendsen barostat (Berendsen et al., 1984). Then, a total of 100 ns production MD simulations were performed for each system using GROMACS 5.0.4 (Van Der Spoel et al., 2005). During the production MD, the temperature and pressure were maintained at 289.15 K and 1 bar by applying the Nosé-Hoover thermostat and the Parrinello-Rahman barostat (Parrinello and Rahman, 1980), respectively. All simulations were carried out with a 2 fs integration time step. For the electrostatic interactions, the particle mesh Ewald (PME) summation method (Essmann et al., 1995) was used and for the Lennard-Jones interactions, the switching function was applied between 9.5 Å and 11 Å to smoothly turn off the interaction energies to zero at the cut-off distance. The convergence of the simulation results was tested by triplicating the MD simulations starting from different initival velocity distribution.

2.2. HeLa AZ-GR reporter cell line

Human luciferase reporter gene cell line AZ-GR was previously generated by Novotna et al. (2012). Briefly, three tandem copies of GRE (GGTACATTTTGTTCT GGTACA GTA CGTCCT GTTCT GGTACAAACT GTTCT) were inserted into pGL4.27 [luc2P/minP/Hygro] vector (Promega) to generate the reporter plasmid (pGL-4.27-GRE) expressing the luciferase reporter under the control of GREs. AZ-GR cells were generated by stable transfection of HeLa human cervix carcinoma cells with reporter plasmid (pGL-4.27-GRE) and are highly specific to glucocorticoids, allowing selective detection of GR activity by luciferase assay (Novotna et al., 2012). HeLa AZ-GR cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovin serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were passaged every 2–3 days, using TrypLE TM Express (1× Phenol red) and Defined Trypsin Inhibitor (1×) (Gibco), and maintained at 37 °C/5% CO_2 in a humidified incubator.

2.3. Methylmercury-dexamethasone exposure procedure

HeLa AZ-GR cells were plated in 96-well microplates (white, clear bottom; Corning) at a concentration of 30.000 cells/well and kept in DMEM medium supplemented with 10% Charcoal Stripped Fetal Bovine Serum (DCC). The following day, cells were exposed to 100 nM MeHg (CH_3HgOH, ALFA, Johnson Matthey, Karlsruhe, Germany). The working MeHg concentration (100 nM) was identified, by Hoechst 33,342 staining, as the highest MeHg concentration not inducing apoptosis in HeLa AZ-GR cells (data not shown). After 6 or, alternatively, 20 h of pre-treatment with MeHg, the cells were exposed to 100 nM Dex (Sigma-Aldrich; Cat No. D1756). The cells were lysed 24 h after Dex exposure in a buffer containing 10 mM Tris (Bio-Rad), 1.99 mM DTT (Sigma) and 2 mM CDTA (Sigma) and the plates were frozen at −80 °C for 24 h. GR activity was measured by luciferase assay.

2.4. Luciferase assay

The luciferase reporter gene assays were used to detect GR-mediated transcriptional effects in the stably transfected HeLa AZ-GR cell line. We measured the luminescence generated by substrate transformation by the GR-dependent luciferase. Before luminometric detection, the plates were thawed and shaken for 60 mins at room temperature. Luminescence was measured (10s integration time) using a plate-reader (Promega) with automatic injection of 100 µL luciferin FlashMix