Full Length Article

**Methylmercury upregulates RE-1 silencing transcription factor (REST) in SH-SY5Y cells and mouse cerebellum**

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1. Introduction

The ubiquitous environmental neurotoxin methylmercury (MeHg) readily crosses the blood-brain barrier, through the L-type large neutral amino acid transporter, and induces serious neurological damage (Simmons-Willis et al., 2002). Low concentrations of mercury are able to induce oxidative stress, cell cytotoxicity and to increase the secretion of β-amyloid 1–40 and 1–42, which may lead to neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases (Olivieri et al., 2002). Moreover, an increase of hypoesthesia, ataxia, dysarthria and impairment of hearing and visual change (Ninomiya et al., 1995) has been found in the patients 10 years after the end of methylmercury dispersion from Minamata, on coast of Shiranui sea, as a consequence of a long-term exposure study to low doses. Indeed, observational studies of individuals exposed to high levels of neurotoxin early in life in Minamata, where methylmercury was discharged in waste water showed neurological dysfunction such as cerebellar ataxia, visual
impairment, weakness of extremities, and sensory disturbances (Eto et al., 2010). The brain regions most susceptible to MeHg-mediated injury are the brain cortex and the cerebellum (Fahrion et al., 2012). For the cerebellum, the degeneration seems to occur particularly in cerebellar granule cells (CCGs) (Fujimura and Usuki, 2014, 2015). Interestingly, in experimental animal studies MeHg induces degeneration of the cerebellar cortex, leaving Purkinje cells intact (Fujimura and Usuki, 2014, 2015, Nagashima et al., 1996). Different molecular targets have been associated with MeHg-induced neuronal cell death, including the N-methyl-D-aspartate (NMDA) receptors (Ndountse and Chan, 2008), Ca\(^{2+}\) channels (Gasso et al., 2001), Rho-associated coiled coil-forming protein kinase (ROCK) (Fujimura et al., 2011), and, recently, the suppression of tropomyosin receptor kinase A (TrkA) pathway (Fujimura and Usuki, 2015).

A new interesting target to investigate in MeHg-induced toxicity is the repressor element 1 (RE-1)–silencing transcription factor (REST). Indeed, perturbation in the expression and function of REST is involved in several neurological disorder such as stroke (Formisano et al., 2015b, Formisano et al., 2013), epilepsy (McClelland et al., 2014), Huntington disease (Zuccato et al., 2003), neuropathic pain (Uchida et al., 2010), and neurotoxicity induced by the environmental neurotoxants PCBs (Formisano et al., 2011, Formisano et al., 2014, Formisano et al., 2015a). It is well known that REST, by binding to the RE-1 elements, actively represses a large array of coding and non-coding RNAs, such as miR-2003, all of which have a pivotal role in synaptogenesis, synaptic plasticity, and structural remodeling (Hwang et al., 2014). Thus, in brain ischemia REST translocates in the nucleus, thus repressing the expression and function of REST by binding to the RE-1 elements, actively represses a large array of coding and non-coding RNAs, such as miR-132, all of which have a pivotal role in synaptogenesis, synaptic plasticity, and structural remodeling (Hwang et al., 2014).

Since we hypothesized the involvement of REST in methylmercury-induced neurotoxicity, we examined the effects of MeHg treatment on REST expression and its role in MeHg-induced neurotoxicity in neuroblastoma SH-SYSY cells and in mouse cerebellum. Our in vitro analyses revealed that REST and histone H4 protein deacetylation are involved in MeHg-induced neurotoxicity; consistently, our in vivo experiments indicate that the subcutaneous administration of MeHg (10 mg/Kg/day) in mice leads to increased levels of REST protein expression in the cerebellum, a region highly vulnerable to MeHg-induced neurotoxicity.

2. Material and methods

2.1. Drug and chemicals

Methylmercury (II) chloride (MeHg) (cod: 442534 stock solution 100 μM) and trichostatin A (TSA) (cod: T8552 stock solution 100 μM) both obtained from Sigma–Aldrich (St. Louis, MO) were dissolved in water with NaHCO\(_3\) at 25 mM (Zimmermann et al., 2014), and with dimethyl sulfoxide (DMSO), respectively. Culture media and sera were purchased from Invitrogen (Milan, Italy). For in vivo experiments, MeHg was conjugated with cysteine (Cys) and its reaction was confirmed by Ellman’s reaction (Zimmermann et al., 2014). All chemicals were diluted in cell culture medium. In all experiments, the control group (CTL) was treated with vehicle: water with NaHCO\(_3\) (25 mM) for MeHg and DMSO (0.1%) for TSA.

2.2. Cell Cultures and siRNA transfections

Human neuroblastoma SH-SYSY cells (IRCCS Azienda Ospedaliere Universitaria San Martino-IST-Instituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) were grown as previously described (Formisano et al., 2014). After 24 h of cell seeding, MeHg at 0.1–3 μM was added to Dulbecco’s Modified Eagle Medium (DMEM) medium containing 1% fetal bovine serum (FBS) for 6–36 h. After cell seeding and before MeHg exposure (1 μM/24 h), cells were pre-treated with TSA (10–50 nM) for 2 h. For REST synthetic small interfering RNA (siRNA) transfection, cells were transfected with siRNA against REST (siREST) and negative control siRNA (siCTL) 24 h before MeHg treatment (1 μM/24 h). The transfection was performed with HiPerFect Transfection Reagent (Qiagen) in accordance with the manufacturer’s protocol. siREST (20 nM) was transfected into SH-SYSY cells as previously reported (Formisano et al., 2015a). The sequences of siRNA for REST were as follows: forward 5’-GAUGGAGGUGGCCGAGAATT-3’ and reverse 5’-UAUCUGGACUCCUCUCAUGT-3’ (NM_005612). For siCTL All Stars Negative Control siRNA (Qiagen) (cod: 1027280) was used. siRNA transfection efficiency was 60% for SH-SYSY (data not shown). Finally, cells were exposed to MeHg (1 μM) for 24 h. They were then plated in 24-multiwell plates at a density of 2 × 10\(^4\) for the MTT assay, annexin V- and propidium iodide (PI) staining, and in 100 mm well plates at a density of 10 × 10\(^5\) for quantitative reverse-transcription polymerase chain reaction (RT-PCR), Western blot, and immunoprecipitation analyses.

2.3. Determination of cell viability

Cell viability was evaluated by MTT assay (Guida et al., 2014) under various experimental conditions: (1) cells were treated with MeHg at increasing concentrations of 0.1–3 μM for 24 h; (2) cells were treated with MeHg at 1 μM for 6–36 h; (3) cells were transfected with siREST and siCTL or pre-treated with TSA (10–50 nM) for 2 h, and, after 24 h, they further incubated with 1 μM MeHg for 24 h.

2.4. Annexin V/Propidium iodide staining

Experiments were performed as previously reported (Formisano et al., 2015a, Guida et al., 2014). Briefly, cells were treated with MeHg either at 0.1–3 μM for 24 h or at 1 μM for 6–36 h. In other experiments, cells were transfected with siREST and siCTL and, 24 h later, treated with 1 μM MeHg for 24 h. After MeHg exposure, they were washed with ice-cold PBS and collected on ice. Apoptosis and necrosis were evaluated by staining the cells with annexin V and propidium iodide (PI), respectively, for 30 min in ice-cold PBS containing both dyes and after that, they were suspended for deposition on slides. A negative sample was acquired for control staining. The analysis was performed on 10 fields for each experimental condition. In addition, the cells were at a concentration of 150,000/ml, and the analyses were performed on 35,000 cells for each experimental condition.

2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA extraction and cDNA synthesis from SH-SYSY cells and quantitative real-time PCR were performed as previously reported (Formisano et al., 2015a, Guida et al., 2014). Samples were amplified simultaneously in quadruplicate in a one-assay run, and the threshold cycle (C\(_t\)) value for each experimental group was determined. Normalization of the data was performed using ribosomal protein L19 (L19). To evaluate differences in mRNA content between groups, normalized values were entered into the