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Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury

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ABSTRACT

Methylmercury (MeHg) is well-known for causing irreversible damage in the central nervous system as well as a risk factor for inducing neuronal degeneration. However, the molecular mechanisms of MeHg-induced neurotoxicity remain unclear. Here, we investigated the effects and possible mechanisms of MeHg in the mouse cerebrum (*in vivo*) and in cultured Neuro-2a cells (*in vitro*). *In vivo* study showed that the levels of LPO in the plasma and cerebral cortex significantly increased after administration of MeHg (50 μg/kg/day) for 7 consecutive weeks. MeHg could also decrease glutathione level and increase the expressions of caspase-3, -7, and -9, accompanied by Bcl-2 down-regulation and up-regulation of Bax, Bak, and p53. Moreover, treatment of Neuro-2a cells with MeHg significantly reduced cell viability, increased oxidative stress damage, and induced several features of mitochondria-dependent apoptotic signals, including increased sub-G1 hypodiploids, mitochondrial dysfunctions, and the activation of PARP, and caspase cascades. These MeHg-induced apoptotic-related signals could be remarkably reversed by antioxidant NAC. MeHg also increased the phosphorylation of ERK1/2 and p38, but not JNK. Pharmacological inhibitors NAC, PD98059, and SB203580 attenuated MeHg-induced cytotoxicity, ERK1/2 and p38 activation, MMP loss, and caspase-3 activation in Neuro-2a cells. Taken together, these results suggest that the signals of ROS-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic pathways that are involved in MeHg-induced neurotoxicity.

Abbreviations: LPO, lipid peroxidation; PARP, poly (ADP-ribose) polymerase; NAC, *N*-acetylcysteine; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; GSH, glutathione.

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1. Introduction

Methylmercury (MeHg) is a highly lipophilic and toxic environmental contaminant. It has become an important public health problem in humans because of the growing evidence of its contamination of the human food chain, such as in fish, shellfish, and other aquatic mammals (Clarkson et al., 2003; U.S. Environmental Protection Agency (EPA, 1997). MeHg, which easily crosses the blood–brain barrier (BBB), is a potent neurotoxin interfering with the brain and nervous system (the primary target tissue for the toxic effects of MeHg), and causes irreversible neuropathophysiological disorders in mammals (Li et al., 2010; Rice and Barone, 2000). It has been clinically reported that after the MeHg disasters in Japan and Iraq, adults as well as infants and children developed severe brain dysfunction and damage (Amin-Zaki et al., 1981; Marsh, 1987). Although a previous study by our group indicated that exposure of mice to MeHg, which may be similar to the dose by ingestion in MeHg-contaminated areas, could cause abnormal neural function (Huang et al., 2008), the signaling mechanisms of MeHg-induced neurotoxic effects are not yet understood.

Oxidative stress has been implicated in a wide variety of biological reactions such as cell death or central nervous system damage (Cuello et al., 2010; Tamm et al., 2006). Exposure to MeHg (1–10 μM) has been reported to induce toxic effects by production of oxidative stress, which alters cellular function and eventually results in pathophysiological injury and cell death in various cells, including neuronal cells (Garg and Chang, 2006; Yin et al., 2007). Some studies have also indicated that MeHg disrupts cellular redox homeostasis and/or antioxidant enzymes and the mitochondrial electron transport chain, via excessive generation of ROS (Sarafian, 1999; Yee and Choi, 1996), thus reducing the mitochondrial inner membrane potential by altering calcium homeostasis (Levesque and Atchison, 1991; Sirois and Atchison, 2000). Moreover, MeHg-induced oxidative stress can inhibit glutamate uptake of astrocytes, while stimulating glutamate over efflux, which results in an excessive level of synaptic glutamate and disrupts glutamine/glutamate cycling (Aschner et al., 2007; Yin et al., 2007). These undesirable MeHg-induced biological processes ultimately lead to neuronal dysfunction and cell death and suggest an association with progressive neurodegenerative diseases (Larkfors et al., 1991; Mutter et al., 2007).

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that mediate a critical role of signal transduction in mammals. MAPKs, which can be subdivided into ERK1/2, JNK, and p38 protein, are activated by modulation of important cellular functions, including proliferation, differentiation, or response to environmental stimuli such as apoptosis (Chang and Karin, 2001; Cowan and Storey, 2003). It has been proposed that deviation from the precise regulation of MAPK signaling pathways could cause the development of human neurodegenerative diseases (Kim and Choi, 2010). ROS-induced oxidative stress has been demonstrated to activate members of the MAPKs via phosphorylation (El-Najjar et al., 2010; Navarro et al., 2006), and it is also implicated in cellular injuries or apoptosis during neurodegenerative disorders (Loh et al., 2006). Recently, oxidative stress-induced ERK/p38 activation has been identified in mammalian cell deaths caused by exposure to toxic chemicals (Martin and Pognonec, 2010; Navarro et al., 2006). However, the mechanism underlying the effect of MeHg-induced oxidative stress in neuronal cells contributing to apoptosis is not clear.

In this study, we attempted to explore the role of oxidative stress-mediated signaling in neuronal cell death by investigating whether ROS generation, mitochondrial dysfunction, caspase cascades, and ERK/p38-MAPK activation are involved in MeHg-induced apoptosis in Neuro-2a cells. Moreover, the use of the potent antioxidant *N*-acetylcysteine (NAC) at different stages confirmed the involvement of major molecules in signaling pathways. We also explored whether exposure to MeHg (50 μg/kg), which mimics the possible exposure dose in humans in MeHg-contaminated areas, and has been reported to cause the neurophysiological dysfunction (Huang et al., 2008), would generate LPO, deplete GSH levels, and alter apoptotic-related gene expressions in the cerebral cortex of mice.

2. Materials and methods

2.1. Cell culture

Murine neuroblastoma cell line: Neuro-2a (CCL-131, American Type Culture Collection, Manassas, VA, USA) was cultured in a humidified chamber with a 5% CO_2–95% air mixture at 37 °C and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA), and all assays were conducted within 10–15 cell passages after unfreezing the cells from the liquid nitrogen (to keep the best morphological and biological characteristics).

2.2. Cell viability

Neuro-2a cells were seeded at 2×10^4 cells/well in 96-well plates and allowed to adhere and recover overnight. The cells were transferred to fresh media and then incubated with MeHg (1–5 μM; Sigma-Aldrich, St. Louis, MO, USA) in the absence or presence of NAC (1 mM) or specific MAPK inhibitors (20 μM, Sigma-Aldrich) for 24 h. After incubation, the medium was aspirated and fresh medium containing 30 μL of 2 mg/mL 3-(4, 5-dimethyl thiazol-2-yl-)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in dimethyl sulfoxide (100 μL; Sigma-Aldrich). Absorbance at 570 nm was measured using a microplate reader (Bio-Rad, model 550, Hercules, CA, USA).

2.3. Determination of reactive oxygen species (ROS) production

ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe: 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Inc., Eugene, OR, USA), as described by Chen et al. (2010). In brief, cells were coincubated with 20 μM DCFH-DA at 37 °C. After incubation with the dye, cells were resuspended in ice-cold phosphate buffered saline (PBS) and placed on ice in a dark environment. The intracellular peroxide levels were measured by flow cytometer (FACScalibur, Becton Dickinson, Sunnyvale, CA), which emitted a fluorescent signal at 525 nm. Each group was acquired with more than 10,000 individual cells.

2.4. Analysis of intracellular GSH contents

Neuro-2a cells were seeded at 2×10^5 cells/well in a 24-well plate and allowed to adhere and recover overnight. The cells were transferred to fresh media and incubated with MeHg in the absence or presence of NAC (1 mM, Sigma-Aldrich) for 24 h. Then, cells were washed twice with PBS, and then a new medium which contained 60 μM monochlorobimane (mBCL, a sensitive fluorescent probe, Sigma-Aldrich) was added and incubated for further 30 min at 37 °C. After loading the culture cells with mBCL, the supernatants were discarded, cells were washed twice with PBS, and the measurement of the intracellular GSH levels was performed as described previously (Yen et al., 2007).

2.5. Flow cytometric analysis of sub-G1 DNA content

Cells were seeded (in the same manner as for intracellular GSH analysis) and incubated with MeHg. After 24 h incubation, the cells were detached, collected, and washed with PBS, and the analysis of sub-G1 DNA content was performed as described previously (Lu et al., 2011). The cells were subjected to flow cytometry analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified as apoptotic. The sample of each group was collected with more than 10,000 individual cells.

2.6. Determination of mitochondrial membrane potential (MMP)

Cells were seeded (in the same manner as for intracellular GSH analysis) and exposed to MeHg in the absence or presence of NAC (1 mM) or specific MAPK inhibitors (20 μM, Sigma-Aldrich). After 6 h or 24 h incubation, cells were loaded with 100 nM 3,3′-di-hexyloxacarbocyanine iodide ($DiOC_6$, Molecular Probes, Inc.) for 30 min at 37 °C, and then trypsinized, collected, and washed twice with PBS. MMP was analyzed by FACScan flow cytometer (excitation at 475 nm and emission at 525 nm, Becton Dickinson) (Chen et al., 2006).