Research Report

Effects of low level of methylmercury on proliferation of cortical progenitor cells

Mingyu Xu¹, Chonghuai Yan¹, Ying Tian¹, Xiaobing Yuan², Xiaoming Shen²,*

¹XinHua Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai Institute for Pediatric Research, Shanghai Key Laboratory of Children's Environmental Health, Shanghai 200092, China
²Institute of Neuroscience and State Key Laboratory of Neurobiology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

ABSTRACT

Methylmercury (MeHg) is a potent environmental neurotoxin that shows toxicity to developing central nervous system (CNS), causing brain damage in children even at low exposure levels. However, the mechanisms for its effect on CNS are not well understood. In current study, primary cultures of progenitor cells from embryonic cerebral cortex were used as a model system to study the potential effect and the underlying mechanism of MeHg on neural progenitor cells. Results showed that, in cultured cortical progenitor cells, 48-h exposure to low-level of MeHg (at 2.5 nM, 5 nM and 50 nM, respectively) caused G1/S cell cycle arrest in a dose-dependent manner without inducing cell death. Interestingly, the expression of cyclin E, which promotes G1/S transition, but not cyclin D1 and CDK2, was selectively downregulated by exposure of MeHg. In addition, low-level of MeHg inhibited the maintenance of ERK1/2 phosphorylation, possibly by abolishing the late phase ERK1/2 activation induced by bFGF. Thus, MeHg may induce proliferation inhibition and cell cycle arrest of neural progenitor cells via regulating cyclin E expression and perturbing a pathway that involves ERK1/2.

1. Introduction

Methylmercury (MeHg) is an environmentally persistent pollutant. Humans typically encounter MeHg from eating fish and seafood which contain trace amount of MeHg, and low-level exposure is essentially ubiquitous. Clinical findings in victims of the Japanese and Iraqi outbreaks have disclosed pronounced susceptibility of the developing central nervous system to this environmental pollutant (Bakir et al., 1973; Eto, 1997). Previous laboratory studies have also demonstrated that the developing brain is particularly vulnerable to MeHg toxicity (Ferraro et al., 2009; Onishchenko et al., 2007; Stringari et al., 2008).

The mechanisms underlying the sensitivity of developing brain to MeHg exposure can be attributed to perturbation of the highly regulated processes associated with brain development, including the rapid and coordinated cell proliferation, differentiation and migration. Progenitor cell proliferation is a key stage for neurogenesis, and cell-cycle parameters also affect rates of neuronal generation. Extrinsic factors that affect progenitor proliferation may interfere with cortical cytoarchitecture. Neuroanatomical study revealed that high dose of
MeHg prenatally exposure can cause decreased cell number and abnormal brain cytoarchitecture (Faustman et al., 2002). However, it is shown by numerous studies that MeHg levels in the cord blood of pregnant women is fairly low nowadays (Bjornberg et al., 2005a), and this raises a concern about the effect of very low levels of MeHg, particularly in nanomolar range, on neurogenesis.

Recent studies have pointed to the selective detrimental effects of MeHg on neurogenesis (Burke et al., 2006; Falluel-Morel et al., 2007). These studies showed that MeHg induced acute inhibition of neuronal proliferation and disturbed the cell cycle progression. However, the molecular mechanisms of the chronic effect at sub-toxic exposure levels remain unknown. Cell cycle progression can be blocked at the G1 checkpoint in response to both the intracellular and extracellular cues. The transition from G1 into the S phase of the cell cycle is controlled by cyclin-dependent kinases (CDKs) complexes. In mid/late G1 stage, cyclin E is induced and forms complexes with CDK2, whose activity appears to be essential for entrance of the S phase (Dehay and Kennedy, 2007). Previous studies demonstrated that MeHg exposure acutely disrupted G1/S transition through reducing cyclins such as cyclin E, cyclin D1and cyclin D3 in hippocampus cells (Falluel-Morel et al., 2007).

Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is also one of key molecules in growth factor signaling. The p42 and p44 ERKs of MAPK family have been shown to regulate a wide range of cellular responses and have particularly well-defined roles in cell proliferation. ERK activation acts at several levels to increase the activity of CDKs in late G1 phase and sustained ERK activation is required for cell proliferation (Chambard et al., 2007). Interestingly, previous study showed that MeHg significantly decreased NGF-induced ERK1/2 phosphorylation after 2.5-min exposure in PC12 cells (Parran et al., 2004).

In this study, we investigated the chronic effects of low-level MeHg on cerebrcortical neurogenesis using a primary culture of cortical progenitor cells derived from embryonic rats. We analyzed particularly the consequences of MeHg exposure on the proliferation of these cultured progenitor cells and signaling pathways that are essential for cell proliferation, in the hope of elucidating the mechanisms by which MeHg causes neurodevelopmental damage. We found that low levels of MeHg suppressed the proliferation of neuronal precursors without affecting their survival. Moreover, MeHg inhibited the ERK signaling pathway and cyclinE was a sensitive target of MeHg.

### 2. Results

#### 2.1. Effects of low-level MeHg exposure on cell viability

To determine whether low-level MeHg affects cell viability, cortical progenitor cultures were incubated with 2.5 nM–50 μM MeHg for 48 h. Cell death induced by MeHg in the culture was evaluated by MTT assay. We observed a dose-dependent decrease in cell viability (Fig. 1). The increase in the cell death upon exposure to MeHg at higher concentrations (500 nM, 5 μM and 50 μM) was statistically significant (p<0.01) compared to the negative (untreated) control or MeHg at lower concentrations (2.5 nM, 5 nM and 50 nM). Thus, low-level MeHg (2.5 nM, 5 nM and 50 nM) did not cause significant occurrence of cell death.

#### 2.2. Effects of low-level MeHg exposure on cell apoptosis

Cortical progenitor cultures were treated with 2.5 nM–50 μM MeHg for 48 h and cell apoptosis was analyzed by immunochemistry. The levels of activated (cleaved) caspase-3, a major mediator of cell apoptosis, and Hoechst/PI double staining were assessed. A statistically significant increase (p<0.01, p<0.05) in the percentage of activated capase-3-positive cells was observed upon treatment with 500 nM, 5 μM and 50 μM MeHg. These increases were statistically different from responses to low concentrations (2.5 nM, 5 nM, 50 nM). In contrast, low-level MeHg did not induce significant increase in cell apoptosis (Fig. 2A, B, C).

#### 2.3. Low-level MeHg exposure reduces proliferation of cortical progenitor cells and induces cell cycle arrest

To directly evaluate the effect of MeHg on the proliferation of cortical progenitor cells, we assayed the cell incorporation rate of BrdU, a thymidine analog that could be incorporated into the genomic DNA as cells progress through S phase and was thus widely used for evaluating cell proliferation. The cultures were incubated with BrdU during the last 4 h of MeHg treatment to label proliferating progenitor cells. The result showed a significant reduction (p<0.01) in the proportion of BrdU-incorporated cells with 48 h exposure of low-level MeHg (Fig. 3), suggesting a reduction in cell-cycle entry in these cells. The decrease in the number of BrdU-positive cells suggests that MeHg may induce cell cycle arrest and inhibit cortical progenitor cells to enter the S-phase.

#### 2.4. MeHg selectively reduces the expression of cyclin E

Considering the decrease in BrdU labeling, we assessed the effects of MeHg on cell cycle regulatory proteins involved in
G1/S transition. Cyclin E is an essential positive G1 regulator that binds to CDK2 during the latter half of the G1 phase to activate its kinase activity and promote the transition into S phase. Consistent with the idea that MeHg inhibits the entry of S phase, Western blotting analysis with protein extracts of the cultures showed that the protein level of cyclin E, the key cyclin protein regulating G1/S transition was down-regulated in a dose-dependent manner (p<0.01) in progenitor cells after 48 h of MeHg exposure (Fig. 4A, B). In contrast, cyclin D1 and CDK2 protein levels were not altered under same treatments (Fig. 4A, C, and D). These data suggest that MeHg may inhibit the proliferation of cortical progenitor cells and induce cell cycle arrest by selectively downregulating the key G1 regulator cyclin E.

Fig. 2 – Effect of MeHg on cell apoptosis. Neural progenitor cells were isolated from the cortex of E13 rat pups and exposed to 2.5 nM, 5 nM, 50 nM, 500 nM, 5 μM and 50 μM MeHg in culture for 48 h. (A,B) Immunocytochemistry was performed with an anti-activated caspase-3 antibody (green). There were no differences in the level of cleaved caspase 3 between low-level MeHg exposure groups (2.5 nM, 5 nM and 50 nM) and the control, whereas there was statistically significant increase in cultures exposure to high-level of MeHg (500 nM, 5 μM and 50 μM). Each value presented is the mean ± S.E.M. (n>3). *, **Significantly different from the untreated control using one-way ANOVA with Student–Newman–Keuls method (*p<0.05, **p<0.01). TOTO-3 nuclear staining (red). (C) Hochst/PI double staining.
Fig. 3 – Effect of MeHg on the proliferation of neural progenitor cells. Neural progenitor cells were isolated from the cortex of E13 rat embryos and exposed to 2.5 nM, 5 nM and 50 nM MeHg in culture for 48 h. The cultures were exposed to BrdU during the last 4 h of MeHg treatment. BrdU incorporating cells were positively stained by the anti-BrdU antibody (green). The percentage of BrdU-positive cells is shown (\(n>3\), mean ± SEM). **Significant different from untreated cells using one-way ANOVA with Student–Newman–Keuls method (\(p<0.01\)). TOTO-3 nuclear staining (red).

Fig. 4 – Effect of MeHg on cyclin E expression. The cellular proteins were extracted 48 h after treatment with 2.5 nM, 5 nM and 50 nM MeHg. (A) Western blot analysis of cyclin E, cyclinD1 and CDK2 protein levels. (B, C, D) Densitometric quantification of the bands shown in (A). The relative intensity of the control (untreated) was regarded as 100%. Each value presented is the mean ± S.E.M. (\(n>3\)). The cyclin E intensities of the treated cells differ significantly from the control. But the intensities of cyclinD and CDK2 do not differ significantly between treated cells and the control. **Significant difference from the untreated cells using one-way ANOVA with Student–Newman–Keuls method (\(p<0.01\)). β-Actin was used as an protein loading control.
2.5. Inhibition of ERK1/2 activation is involved in the reduced proliferation of cortical progenitor cells by MeHg

Many studies have shown that sustained ERK activation is essential for growth factor-induced cell cycle progression (Roovers and Assoian, 2000). Recent evidences indicate that ERK activation throughout G1 phase is required for the cell entry of S phase (Yamamoto et al., 2006). To examine the potential role of ERK signaling pathway in MeHg-induced cell cycle arrest, we investigated the levels of phosphorylated and total ERK 1/2. After 48 h of exposure to MeHg, ERK1/2 phosphorylation was reduced in a dose-dependent manner (Fig. 5A, B). As shown in Fig. 5B, at the level of 50 nM, MeHg reduced the ERK1/2 phosphorylation level by approximately 48% compared with the control (p < 0.01). In contrast, the total ERK 1/2 protein level was not affected under these treatments.

To further determine how MeHg affects the ERK signaling pathway, the phosphorylated and total ERK1/2 were examined at different time points after bFGF stimulation, including 0 min, 30 min, 1 h, 6 h, 12 h, 18 h and 24 h, with or without treatment with 50 nM MeHg. We observed that activation of ERK1/2 was induced rapidly upon stimulation with bFGF with or without MeHg treatment, and there was sustained activation of ERK1/2 that peaked at 1 h after bFGF stimulation and then decreased in both groups. However, in cells exposed to 50 nM MeHg, the level of phosphorylated ERK1/2 decreased more significantly at 12 h, 18 h and 24 h compared with that in the control group (Fig. 6). These results suggest that exposure of cortical progenitor cells to MeHg did not affect the early phase activation of ERK1/2 by bFGF, but inhibited the maintenance of bFGF-induced ERK1/2 phosphorylation. Considering the essential role of ERK signaling in cell cycle progression, these results suggest that MeHg may inhibit the entry of S phase by suppressing the late phase ERK activation induced by bFGF in these cultured progenitor cells.

3. Discussion

MeHg has been a health hazard for more than 50 years because of its toxicity to the central nervous system. In the present study we used primary cultures of neural progenitor cells from cortical tissues of E13 rat as a model system to investigate the effects of MeHg on cortical neurogenesis. Previous studies have shown that neural progenitor cells are highly sensitive to acute exposure of MeHg (Burke et al., 2006). However, the low levels of MeHg reported in the cord blood of pregnant women raise a concern about the effect of very low levels of MeHg on developmental neuronal tissues. Therefore in this study we chose to use concentrations at 2.5 nM to 50 nM, which are even lower than that detected in the umbilical cord blood of pregnant women in many countries (Bjornberg et al., 2005b; Tamm et al., 2008). We focused on the chronic effects of these subtoxic levels of MeHg. We found that 48 h exposure to very low levels of MeHg, which mimic the environmental exposure, inhibited the proliferation of cultured neural progenitor cells without inducing cell death with concurrent selective down-regulation cyclin E protein level and inhibition of ERK signaling cascade.

MeHg was shown to elicit cell death in neuronal cell lines via both necrotic and apoptotic pathways (Nishioku et al., 2000). Caspase-dependent apoptotic pathways were shown to be involved in MeHg-induced apoptosis (Fujimura et al., 2009; Tamm et al., 2006). We used immunocytochemical assays with antibody against activated caspase-3 and Hoechst/PI double staining to identify that the cultured neural progenitor cells did not undergo apoptosis after 48 h of MeHg exposure at low levels (2.5 nM, 5 nM, and 50 nM).

Neuropathological studies indicated that MeHg exposure reduced cell number and brain weight in both humans and experimentally exposed animals (Choi, 1989; Geelen et al., 1990). These observations suggest that MeHg, as a neurotoxin to the developing brain, could affect cell proliferation, one of the main cellular events during embryonic development. Observed reduction in cell number without significant cell necrosis or apoptosis also suggested that MeHg plays a role in suppressing cell proliferation (Faustman et al., 2002). The processes of cell proliferation and cell cycle progression have been extensively studied following MeHg treatment in vivo and in vitro (Burbacher et al., 1990; Gribble et al., 2005; Howard and Mottet, 1986; Lewandowski et al., 2002). Most studies focused on midbrain neurogenesis. However, data regarding cerebro-cortical neurogenesis at low and environmentally relevant concentrations remained limited. We demonstrated here for the first time that proliferation of neural progenitor cells is reduced by MeHg exposure at nanomolar concentrations,
shown by a reduction of BrdU-positive cells 48 h after MeHg exposure. Results from this study support the current theory of MeHg neurotoxicity that cell cycle arrest is a possible cause of decreased neuronal cell numbers previously observed in the brains of infants and laboratory animals exposed to MeHg in utero.

This result emphasizes the need to examine the impacts of MeHg on cell cycle regulators to better understand the molecular mechanisms of growth arrest in cortical neurogenesis in response to MeHg exposure. In cortical progenitors, proliferation and growth arrest are highly regulated by a balanced arrays of extrinsic and intrinsic signals. Exquisite control of the cell cycle is essential during embryonic development, in which slight perturbations can produce extreme consequences. The cell cycle can be divided into four successive phases: M phase (mitosis), in which the nucleus and the cytoplasm divide, S phase (DNA synthesis), in which the DNA in the nucleus is replicated, and two gap phases, G1 and G2. The G1 phase is a critical stage, allowing responses to extracellular signals that induce either entry to another round of cell division or withdrawal from the cell cycle (Zetterberg et al., 1995). In this study, the BrdU incorporation assay revealed that low-level MeHg exposure induced G1 cell cycle arrest in a dose-dependent manner. This observation is in agreement with previous findings (Burke et al., 2006). Previous in vitro studies found that MeHg caused cell cycle arrest involving p21 (Mendoza et al., 2002) and p53 (Gribble et al., 2005). But the mechanism is not well understood. The transition from one phase of the cell cycle to the next is controlled by cyclin-CDK complexes which ensure that all events of the cell cycle progress in the correct order. Regulation of early G1 phase depends on cyclin D-dependent kinases and the late G1/S transition critically depends on cyclin E/CDK2 complexes, which phosphorylate retinoblastoma protein, allowing S-phase gene activation (Sherr and Roberts, 1999). It was reported recently that MeHg exposure rapidly affected cyclin E expression. Exposure to 3 μM MeHg for 6 h induced a marked reduction in levels of cyclin E in cortical cultures (Burke et al., 2006). A single exposure to MeHg (5 μg/gbw) in 7-day-old rat pups for 24 h elicited cyclin E degradation, along with reduction of cyclin D1 and cyclin D3 levels in hippocampus (Ferraro et al., 2009). These studies demonstrated that cyclin E is a selective target of MeHg and its downregulation may contribute to MeHg-induced cell cycle arrest. Consistent with these observations, our study revealed that MeHg selectively reduced cyclin E expression in neural progenitor cells after 48 h of low-level MeHg exposure. But no significant changes were observed in levels of cyclin D1 and CDK2. This suggests that cyclin E is the specific neurotoxicity target of chronic MeHg exposure. Studies with other exposure model have also reported that cyclin E maybe a new locus of MeHg toxicity (Burke et al., 2006) with an acute, micromolar level of exposure. As we know, the effect of MeHg may vary with dosage, duration of exposure and the model system used. Nanomolar levels of MeHg exposure for 48 h caused the same effect on cyclin E, a key cell cycle protein, as with an acute exposure to 3 μM MeHg. The result of this study raised more concern over cellular toxicity caused by environmentally relevant, very low level of MeHg exposure.

This result highlights the need to specifically examine the impacts of MeHg on intracellular signaling pathways in order to better understand the molecular mechanisms of cell cycle arrest. The Ras/Raf/MEK/ERK signaling cascade is one of the key signaling pathways that mediate extracellular growth stimulus.
Recent evidences indicate that sustained ERK activation during G1 phase is required for the entry of S phase (Jones and Kazlauskas, 2001; Yamamoto et al., 2006). The formation of the cyclin E/CDK2 complex is also regulated by ERK (Chambard et al., 2007). Interestingly, ERK signaling pathway has been shown to be affected by MeHg. In PC12 cell model, 0.1–3 μM MeHg inhibited ERK activation after 2.5 min of exposure and reduced the neurite outgrowth (Parran et al., 2004). A phospho-specific antibody was used in this study to examine the effects of MeHg on the phosphorylation status of ERK1/2, which is an index of cell proliferation. Exposure to MeHg for 48 h decreased phosphorylation of ERK in a dose-dependent manner. To elucidate how MeHg affects ERK activation, dynamic expression of phosphorylated form of ERK1/2 were examined. The phospho-ERK1/2 was activated 30 min after bFGF was given, peaked at 1 h and then declined slowly with or without MeHg treatment. But in cultures exposed to 50 nM MeHg, the level of phospho-ERK1/2 was significantly downregulated after 12 h compared with the control. These results suggest that MeHg does not affect activation of ERK signaling pathways, but rather, promotes degradation of phospho-ERK1/2. MeHg was known to induce the release of free radicals (Aschner et al., 2007; Mori et al., 2007), which could also be one of the mechanisms that caused the damage.

In conclusion, chronic exposure to subtoxic, nanomolar concentration of MeHg inhibited the proliferation of neural progenitor cells in primary cell cultures. The neurotoxicity by MeHg is concurrent with, and potentially caused by reduction in cyclin E expression and inhibition of cell cycle (G1/ S arrest). The results suggest that downregulation of ERK signaling by MeHg may be involved in cell cycle arrest.

4. Experimental procedures

4.1. Cultures of rat cortical progenitor cells

Rat embryos at day 13 (E13) from Sprague–Dawley (SD) rat were removed under sterile conditions, and cortices from embryos were microdissected, pooled, digested with 0.125% trypsin and mechanically dissociated. Isolated cells were plated on poly-lysine-coated 35 mm plates and cultured with DMEM supplemented with B27, N2 and mitogen bFGF. Cultures were grown lysemically dissociated. Isolated cells were plated on poly-lysine-coated glass coverslips in a 35-mm dish. To detect activated caspase-3 expressions on cultures after 48 h of MeHg exposure, cells on the coverslips were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Cells were immunostained with anti-activated caspase-3 antibody (rabbit polyclonal; Cell Signaling, 1:1000 in 0.3% Triton-X solution) for 24 h at 4 °C. Secondary antibody (Alexa 488 goat anti-rabbit; Chemicon) incubation was carried out in the dark for 1 h at room temperature. TOTO-3 nuclear staining (Invitrogen) was performed as described previously (Chen et al., 2008). To detect the early apoptosis, cells were double-stained for 10 min with 10 μM Hoechst 33342 (Sigma) and 10 μM propidium iodide (PI) (Sigma). After extensive washing in PBS, the coverslips were mounted and examined under an Olympus F1000 confocal system.

4.4. Immunofluorescence with activated caspase-3 antibody and Hoechst/PI double staining

BrdU incorporation assay was performed as previously described (Wojtowicz and Kee, 2006). Briefly, cells (30864 cells/ cm2) were plated onto poly-lysine-coated glass coverslips in a 35-mm dish and were incubated with 10 μM BrdU for 4 h. Afterward, cells were fixed with 4% paraformaldehyde for 20 min and then treated with 2 N HCl for 30 min at 37 °C, followed by treatment with 0.1 M borate buffer (Na2B4O7-H2O, pH 8.5) for 30 min. Cells were then incubated with monoclonal anti-BrdU (mouse monoclonal; sigma, 1:200 in 0.3% Triton-X solution) for 24 h at 4 °C. The secondary antibody incubation (Alexa 488 goat anti-mouse; Chemicon, 1:200) was carried out in the dark for 1 h at room temperature. After incubation, coverslips were washed in PBS, followed by double-distilled water. The coverslips were allowed to air dry in the dark at room temperature and then mounted and examined under an Olympus F1000 confocal system. Images were processed using Adobe Photoshop CS 8.0.

4.5. Bromodeoxyuridine (BrdU) incorporation assay

Western blotting was performed as described previously (Chen et al., 2008). Briefly, cells were lysed in 0.2 ml lysis buffer (0.1 % SDS, 1 % NP-40, 50 mM HEPES, pH 7.4, 2 mM EDTA, 100 mM NaCl, 5 mM sodium orthovanadate, 40 μM PNPP) with 1 % Protease Inhibitor Cocktail Set I (Calbiochem). Lysates were centrifuged at 12,000 rpm for 25 min. Denatured proteins were collected in the supernatant. Samples were separated by electrophoresis in 10–12% SDS-polyacrylamide gel and transferred onto PVDF membrane. Blots were blocked for 1 h at room temperature.
temperature in 5% nonfat milk, followed by overnight incubation at 4°C with the following primary antibodies respectively: anti-Cyclin E (rabbit polyclonal, Santa Cruz, 1:400), anti-Cyclin D1 (rabbit polyclonal, Lab Vision 1:400), anti-CDK2 (rabbit polyclonal, Santa Cruz, 1:400), anti-α-ERK (mouse monoclonal, Chemicon, 1:3000), anti-α-Tubulin (mouse monoclonal, Sigma, 1:3000) and anti-α-Actin (mouse monoclonal, Chemicon, 1:3000). Membranes were rinsed and incubated for 1 h with the following secondary antibodies: peroxidase-conjugated goat anti-mouse, goat anti-rabbit IgG (Bio-Rad, 1:8000). Chemiluminescent detection was performed with the ECL kit (Pierce).

4.7. Statistical analysis

Data were expressed as means ± S.E.M. Statistical significances between groups were assessed by one-way analysis of variance (ANOVA). Student–Newman–Keuls’ multiple comparison test was employed to compare individual means as a post-hoc test. The differences were considered statistically significant when p < 0.01 and p < 0.05.

Acknowledgments

This work was supported by National Natural Science Foundation of China (30872806) and Foundation for Open Projects of Key Laboratory of Neuroscience, Chinese Academy of Sciences (SKLN-2008B04). This work was funded by Shanghai Key Laboratory of Children’s Environmental Health (09DZ2272200, 09DZ2200900).

REFERENCES


