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Methylmercury induces oxidative stress and subsequent neural hyperactivity leading to cell death through the p38 MAPK-CREB pathway in differentiated SH-SY5Y cells

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ABSTRACT

Methylmercury (MeHg) induces site-specific cerebrocortical neuronal cell death. In our previous study using an *in vivo* mouse model, we reported that MeHg-induced cerebrocortical neuronal cell death may be due to neural hyperactivity triggered by activation of kinase pathways. However, the detailed molecular mechanism remained to be completely understood. In this study, we analyzed detailed signaling pathways for MeHg-induced neuronal cell death using *all-trans*-retinoic acid (RA) differentiated SH-SY5Y cells, which show neuron-like morphological changes and express neuron/synapse markers for cerebrocortical neurons. Time course studies revealed that MeHg-induced upregulation of c-fos, a marker of neural activation, preceded neuronal cell death. These results were similar to those observed in a MeHg-intoxicated mouse model. We observed early expression of the oxidative stress marker thymidine glycol followed by activation of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK, and an increase in cAMP response element binding protein (CREB). Investigation of the effects of specific kinase inhibitors revealed that SB203580, a specific inhibitor for p38 MAPK, significantly blocked the upregulation of c-fos and the subsequent neuronal cell death. In contrast, PD98059 and U0126, specific inhibitors for p44/p42 MAPK, showed no effects on MeHg-induced neurotoxicity. Furthermore, the antioxidants Trolox and edaravone significantly suppressed MeHg-induced thymidine glycol expression, p38 MAPK-CREB pathway activation, and neurotoxicity. Altogether, these results suggest that MeHg-induced oxidative stress and subsequent activation of the p38 MAPK-CREB pathway contribute to cerebrocortical neuronal hyperactivity and subsequent neuronal cell death.

1. Introduction

Methylmercury (MeHg) is a ubiquitous environmental pollutant and is widely known to be a neurotoxic compound (Bakir et al., 1973; Takeuchi, 1982). MeHg toxicity involves two characteristic clinical forms in humans, fetal type and adult type Minamata disease. Fetal-type Minamata disease caused by exposure to MeHg *in utero*, is characterized by extensive brain lesions, whereas the adult-type Minamata disease is caused by MeHg exposure in adulthood and causes site-specific brain lesions in the cerebrum and cerebellum. Autopsy studies of the human cerebrum have revealed that these lesions are localized in the deep layer of the cerebral cortex, especially layer IV (Eto and Takeuchi, 1978; Eto et al., 1999). The MeHg-susceptible layer IV cerebrocortical neurons are located in the granular cortex and have been shown to mainly be excitable cells (Jones, 1975). This suggests that excitability may be related to susceptibility to neuronal MeHg intoxication and subsequent damage.

We previously reported that damage caused by MeHg is localized in layer IV of the cerebral cortex in adult mice, especially within the somatosensory cortex (Fujimura et al., 2009b). A follow-up study from our group using cerebrocortical neurons of MeHg-intoxicated adult mice suggested the possibility that MeHg-induced neuronal degeneration is caused by site-specific neural hyperactivity triggered by the activation of mitogen-activated protein kinase (MAPK) and protein kinase A (PKA)/ cAMP response element binding protein (CREB) pathways followed by c-fos upregulation (Fujimura and Usuki, 2017a). In that study, we showed that site-specific upregulation of c-fos, a proper marker for neural activity, preceded neuronal degeneration in layer IV

Abbreviations: MeHg, methylmercury; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; CREB, cAMP response element binding protein; RA, retinoic acid; ELISA, enzyme-linked immunosorbent assay; NF-H, neurofilament triplet H protein; TG, thymidine glycerol

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of the cerebral somatosensory cortex of MeHg-intoxicated mice. However, we were unable to confirm the involved pathway in layer IV of the cerebral somatosensory cortex of MeHg-intoxicated mice by the inhibition study using enzyme specific inhibitors, because these inhibitors could not pass through the blood-brain barrier. In addition, the primary excitable cell culture from the cerebral cortex was so difficult.

Human neuroblastoma SH-SY5Y is a neuronal cell line which is often used as an *in vitro* model for neurotoxicity experiments. SH-SY5Y cells differentiate when exposed to *all-trans*-retinoic acid (RA) to obtain more neuron-like properties, including neurite outgrowth and morphological changes (Påhlman et al., 1984). Therefore, these cells provide the opportunity to mimic neuronal responses (Jantas et al., 2014; Lopes et al., 2017). However, changes in neuronal properties triggered by RA as well as changes triggered by subsequent exposure to MeHg have not yet been comprehensively studied.

In the present study, we investigated the detailed signaling pathways that lead to MeHg-induced neuronal cell death using RA-induced differentiated SH-SY5Y cells as a model of cerebrocortical neuronal cells. Our results demonstrate that RA-induced differentiated SH-SY5Y cells express neuron-specific proteins and similar synapse markers associated with cerebrocortical neurons of the somatosensory cortex. The differentiated SH-SY5Y cells showed upregulation of c-fos, a marker of neural activity, after exposure to MeHg. Furthermore, we report a detailed mechanism of MeHg-induced neural hyperactivity, which we determined using inhibitors of oxidative stress and of kinase pathways upstream of c-fos expression.

2. Materials and methods

2.1. Cell culture and compounds treatment

SH-SY5Y cells (DS Pharma Biomedical, Osaka, Japan) were cultured as described previously (Lopes et al., 2010). In brief, cells were plated in collagen-coated tissue culture plate wells (Sumitomo Bakelite Co., Tokyo, Japan) at a density of 1.0×10^5 cells/cm^2 in DMEM/F-12 supplemented with 10% fetal bovine serum (DS Pharma Biomedical), and 2 mM $_L$-glutamine (Invitrogen, Carlsbad, USA). Neuronal differentiation was induced with 10 μM RA (Wako Chemicals, Osaka, Japan) for 7 days in DMEM/F-12 supplemented with 1% fetal bovine serum and 2 mM $_L$-glutamine. Non-differentiated cells were cultured in the same culture medium but without RA. The differentiated and non-differentiated cells were separately exposed to MeHg chloride (Tokyo kasei, Tokyo, Japan). In this inhibition study, various kinase inhibitors: PD98059, U0126, SB203580, and antioxidants: Trolox and edaravone (Wako Pure Chemicals) were dissolved in dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) or sterilized water, and MeHg was concomitantly administered.

2.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to measure neurofilament triplet H protein (NF-H) and thymidine glycerol (TG) expression as described previously (Fujimura and Usuki, 2015a; Fujimura et al., 2016). In brief, the cells were fixed with 4% paraformaldehyde in PBS for 30 min in 96-well microplates. They were then washed with PBS and subsequently incubated with 5% fetal calf serum (Thermo Fisher Scientific, Suwanee, USA) and 5% milk diluted in PBS (Wako Pure Chemicals) for 2 h. Either anti-NF-H (Cell Signaling Technology, Danvers, USA) or anti-TG antibody (Wako Pure Chemicals) was added, and the plates were then incubated for 1 h at room temperature. The plates were then washed 3 times with 0.05% Tween 20 (Wako Pure Chemicals) in PBS, and the cells were treated with horseradish peroxidase-conjugated anti-mouse IgG (Promega) for 30 min. The plates were washed 3 times with PBS and then incubated with a substrate solution (3,3′,5,5′-tetramethylbenzidine, Sumitomo Bakelite) for 20 min at room temperature. The optical density at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, USA).

2.3. Western blot analysis

Electrophoresis was performed using 10% sodium dodecyl sulfate-polyacrylamide (SDS) gels (Tefco, Tokyo, Japan) to detect low molecular weight proteins (25–60 kDa) and 4–12% gradient SDS gels (Tefco) for IP_3R1 (320 kDa). Western blotting was performed as previously described (Fujimura and Usuki, 2015b, 2017a) using antibodies against tyrosine hydroxylase (TH), c-fos, CREB, phospho-CREB (Ser133), protein kinase B (Akt), phospho-Akt (Ser473), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) (all from Cell Signaling Technology), neuron-specific enolase (NSE) (Medical and Biology Laboratories, Nagoya, Japan), rab3A (Bioss Antibodies, Woburn, USA), synaptophysin (Epitomics, Burlingame, USA), inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1), protein phosphatase 1 (PP1), PP2 A (Chemicon, Temecula, USA) and β-actin (Sigma-Aldrich, St Louis, USA). The proteins were detected using a chemiluminescence system with an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK). Densitometric quantifications of the immunoblots were performed using Quantity One software version 4.6.3 (Bio-Rad Laboratories).

2.4. Cell viability assay

Cell viability was evaluated using a cell viability kit (Promega, Madison, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), which is a substrate for intracellular dehydrogenases (Fujimura et al., 2009a; Fujimura and Usuki, 2015b). We confirmed that MeHg does not directly interfere with the assay (data not shown).

2.5. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). We performed statistical analysis using a one-way ANOVA followed by Dunnett's multiple comparison tests. Statistical analyses were considered significant when p was less than 0.05.

3. Results

3.1. Characterization of RA-differentiated SH-SY5Y cells

Phase contrast microscopy showed morphological changes in SH-SY5Y cells exposed to RA for 7 days, as shown in Fig. 1a. Exposure to RA for 7 days induced neuron-like properties and neurite outgrowth. Quantitative analysis for neurite outgrowth was performed using an ELISA for NF-H as a neurite-specific protein (Fujimura and Usuki, 2015a). RA exposure for 7 days significantly increased NF-H levels in SH-SY5Y cells (Fig. 1b). RA also increased expression of the neuron-specific proteins NSE and TH as well as the synapse markers IP_3R1, synaptophysin, and rab3A (Fig. 1c, d).

3.2. Cell viability of RA-differentiated and non-differentiated SH-SY5Y cells after MeHg exposure

The cell viability of both RA-differentiated and non-differentiated cells was analyzed 24 h after exposure to MeHg (ranging in concentration from 250 nM to 1250 nM; Fig. 2a). The RA-differentiated cells showed more resistance to MeHg than the non-RA treated SH-SY5Y cells (EC_{50}: 730 nM *vs* 620 nM, respectively). Cell viability was not affected until 6 h after exposure to 750 nM MeHg in both cell types (Fig. 2b). On the basis of these results, a concentration of 750 nM MeHg was adopted for the remaining experiments.