Note

Modulation of Unfolded Protein Response by Methylmercury

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Methylmercury (MeHg) results in cell death through endoplasmic reticulum (ER) stress. Previously, we reported that MeHg induces *S*-mercuration at cysteine 383 or 386 in protein disulfide isomerase (PDI), and this modification induces the loss of enzymatic activity. Because PDI is a key enzyme for the maturation of nascent protein harboring a disulfide bond, the disruption in PDI function by MeHg results in ER stress *via* the accumulation of misfolded proteins. However, the effects of MeHg on unfolded protein response (UPR) sensors and their signaling remain unclear. In the present study, we show that UPR is regulated by MeHg. We found that MeHg specifically attenuated inositol-requiring enzyme 1*α* (IRE1*α*)–x-box binding protein 1 (XBP1) branch, but not the protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcriptional factor 6 (ATF6) branches. Treatment with GSK2606414, a specific PERK inhibitor, significantly inhibited MeHg-induced cell death. These findings suggest that MeHg exquisitely regulates UPR signaling involved in cell death.

Key words methylmercury; endoplasmic reticulum (ER) stress; unfolded protein response; cell death

Methylmercury (MeHg), an environmental pollutant, is a neurotoxin that poses a major risk to human health.[1)] MeHg causes damage to the central nervous system, resulting in Minamata disease.[2,3)] MeHg has a high affinity for the sulfhydryl groups of proteins, facilitating its accumulation in various tissues.[4)] Particularly, MeHg is rapidly absorbed by the gastrointestinal tract and readily diffuses through the blood–brain barrier *via* the L-type large neutral amino acid transporter as MeHg–L-cysteine (Cys) conjugates.[5)] Therefore, the brain is a critical organ for MeHg toxicity. Also, MeHg toxicity is caused by binding of MeHg to the cysteinyl groups of proteins, which in turn interferes with the synthesis of cellular glutathione, thereby leading to oxidative damage.[6)] Alternatively, because MeHg has a high affinity for thiols, its toxicity is dependent on the modifications of covalent bonds (*i.e.*, *S*-mercuration). Thus, the modifications involving the cysteine residues of intracellular proteins caused by MeHg result in the disruption of homeostasis or cell death.

Previously, we demonstrated that both nitric oxide (NO) and MeHg result in protein disulfide isomerase (PDI) dysfunction *via* covalent modifications involving the cysteine residues in active sites of proteins.[7,8)] *S*-Nitrosylation and -mercuration of PDI in active sites promote endoplasmic reticulum (ER) stress and unfolded protein response (UPR). We earlier demonstrated that inositol-requiring enzyme 1*α* (IRE1*α*), a sensor of ER stress that is located within the ER membrane, is also directly modified and regulated by NO.[9)] This modification leads to the inhibition of endonuclease activity, thereby attenuating the splicing of immature *XBP1* mRNA. Because nucleophiles such as NO and MeHg easily react with protein thiols and affect enzymatic activity or function, it is important to determine its molecular targets and to characterize their effects on cells.[10)]

To date, the precise mechanism underlying MeHg-induced cell death remains elusive. Based on the findings of our previous study, we assume that MeHg also affects the condition of UPR sensors during ER stress, particularly in strongly inducing neuronal cell death. The aim of the present study was to elucidate the mechanism underlying cell death *via* a MeHg-induced ER stress/UPR.

MATERIALS AND METHODS

Materials and Antibodies MeHg was purchased from Tokyo Chemical Industry (Tokyo, Japan). GSK2606414 was from Merck Millipore (Billerica, MA, U.S.A.). Antibodies against IRE1*α*, phospho-IRE1*α*, protein kinase RNA-like endoplasmic reticulum kinase (PERK), phospho-PERK, eukaryotic initiation factor 2 (eIF2) *α*, phospho-eIF2*α*, activating transcription factor 6 (ATF6) and *β*-actin were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture Mouse embryonic fibroblasts (MEF) and human neuroblastoma SH-SY5Y cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) heat-inactivated fetal calf serum at 37°C in humidified atmosphere of 5% CO_2/95% air.

Western Blot Analysis Cells were cultured in the indicated medium, harvested, washed with phosphate-buffered saline (PBS), and lysed in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediamine *N,N,N′N′*-tetraacetic acid ethylenediaminetetraacetic acid (EDTA), and 1% NP-40 containing a protease inhibitor cocktail] for 10 min.

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To detect ATF6, the cells were pre-incubated with or without 50 μM of cycloheximide and 5 μM of MG-132 for 3 h and then further incubated for MeHg stimulation. After quantification by using the Bradford assay, the proteins were boiled in a sample loading buffer for 5 min and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 5% non-fat dry milk or bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Then, the membrane was incubated with IRE1α (1 : 5000), phospho-IRE1α (1 : 5000), eIF2α (1 : 5000), phospho-eIF2α (1 : 5000), or β-actin (1 : 5000) antibodies in TBST and then detected by using respective anti-horseradish peroxidase (HRP)-linked secondary antibodies (1: 50000) in TBST. The antibody-reactive bands were visualized by enhanced chemiluminescence (ECL) using the ChemiDoc MP Imaging System (Bio-Rad). Blots were then quantified using ImageJ, and relative ratios were calculated (https://imagej.nih.gov/ij/docs/faqs.html).[11,12]

RNA Extraction and RT-PCR Total RNA was extracted using a TRI reagent. Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) was used to synthesize the cDNA according to the manufacturer's instructions. The cDNA was amplified by using 25 or 30 cycles of PCR. The following primers were used: *XBP1* sense primer, 5′-TTA CGA GAG AAA ACT CAT GGC C-3′; *XBP1* antisense primer, 5′-GGG TCC AAG TTG TCC AGA ATG C-3′; *β-actin* sense primer, 5′-CCT GAC GGC CAG GTC ATC-3′; *β-actin* antisense primer, 5′-GGA CTC GTC ATA CTC CTG-3′. The PCR products were analyzed by agarose gel electrophoresis on a 1.5% gel.

Assessment of Nuclear Morphology Chromosomal condensation was assessed using the fluorescent dye Hoechst 33258. Briefly, cells were stained using 10 μM Hoechst33258 and washed with PBS. All samples were mounted and observed under a fluorescence microscope. The results were expressed as the percentage of cells with condensed nucleus *versus* the total number of cells.[13]

Statistical Analysis All experiments were repeated independently at least three times. The results are expressed as the mean±standard error of the mean (S.E.M.) Statistical comparisons were performed by using ANOVA *post hoc* Bonferroni's test (for multiple groups) using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, U.S.A.).

RESULTS AND DISCUSSION

Previous investigations have elucidated the mechanism of cell death in response to MeHg by inducing ER stress. However, the target molecules and the precise mechanism underlying MeHg-induced cell death remains unclear.[14] We previously showed that PDI is a possible direct target of MeHg, and its modification leads to the attenuation of the enzymatic activity.[8] The dysfunction of PDI results in ER stress and the activation of UPR by sensing the accumulation of immature unfolded proteins in ER lumen. In the present study, we examined the modulation of UPR *via* treatment with MeHg.

During ER stress, *XBP1* mRNA is alternately spliced and activated by IRE1α, which is a UPR component.[15] To address whether the IRE1α–XBP1 branch in UPR is affected by

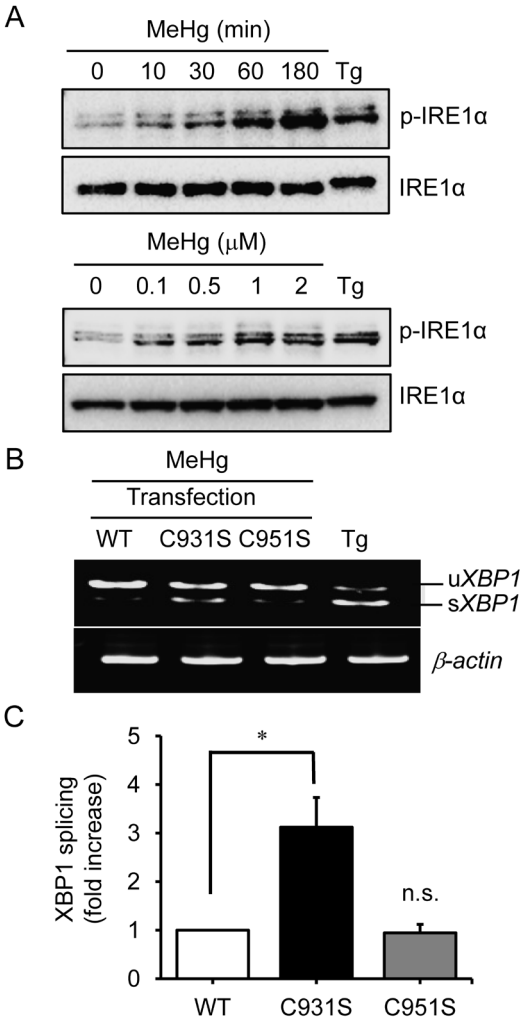

Fig. 1. MeHg Modulated IRE1α–XBP1 Branch of the UPR

(A) MeHg stimulates IRE1α phosphorylation. Cells were treated with 5 μM or varying concentrations of MeHg for indicated periods or exposed to 1 μM thapsigargin (Tg) for 15 min, and then cell lysates were prepared. The lysates were electrophoresed and then immunoblotted with anti-IRE1α or anti-phosphoIRE1α. (B) Attenuation of *XBP1* mRNA splicing by MeHg. IRE1α–null MEF transfected with wild-type (WT) or IRE1α cysteine-to-serine mutants were treated with MeHg for 12 h, and immature *XBP1* mRNA splicing was assessed using RT-PCR. (C) Quantification of the immature *XBP1* mRNA splicing. Values are expressed as the mean±S.E.M. (n=3; *p<0.05: significantly different from MeHg-exposed WT-transfected cells (ANOVA *post hoc* Bonferroni's test), n.s.; not significantly).

MeHg, we evaluated the process of IRE1α phosphorylation in MEF. Figure 1A shows that MeHg results in the phosphorylation of IRE1α in concentration- and time-dependent manners, which in turn suggests that MeHg induces ER stress and activates IRE1α by sensing the accumulation of misfolded proteins in the ER lumen. We previously found that NO elicited ER stress and then promoted IRE1α phosphorylation.[9] Interestingly, the ribonuclease activity of IRE1α was strongly abolished *via* the *S*-nitrosylation of the kinase-extension nuclease (KEN) domain in IRE1α, although phosphorylated IRE1α was detected.[16] Therefore, we consider that MeHg also regulates IRE1α endonuclease activity. Subsequently, we examined the splicing of immature *XBP1* mRNA (a selective substrate) to assess the enzymatic activity of IRE1α. As expected, MeHg did not induce the cytosolic splicing of immature *XBP1* mRNA in MEFs. The IRE1α–null MEFs were transfected with vectors encoding wild-type hemagglutinin (HA)-tagged IRE1α or cysteine mutants.[17] MeHg-induced inhibition of IRE1α en-

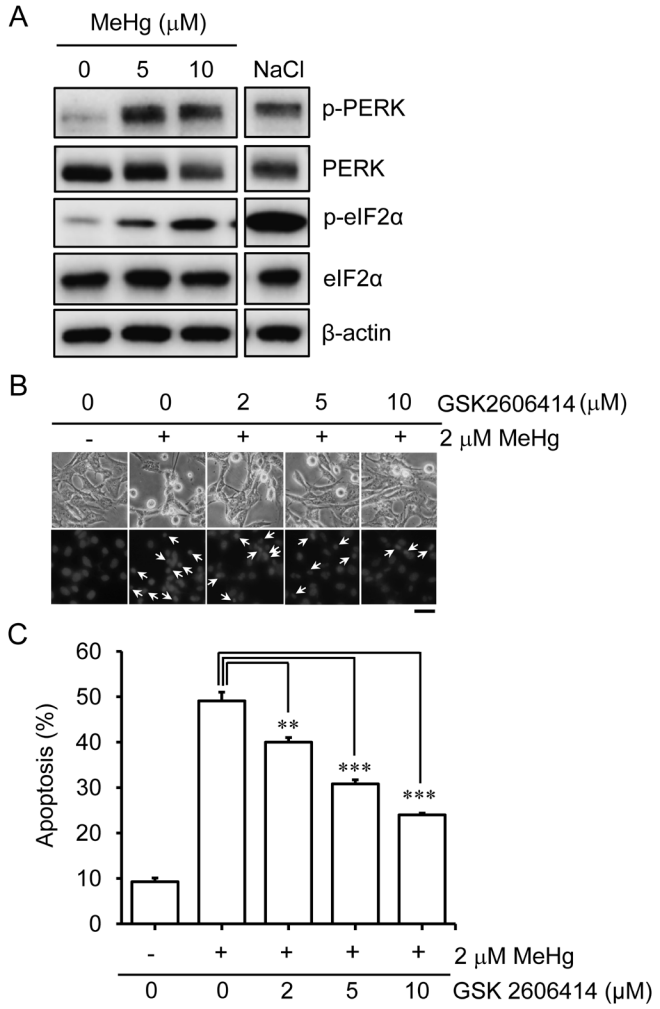

Fig. 2. Involvement of the PERK Branch in MeHg-Induced Apoptosis

(A) MeHg-induced phosphorylation of PERK and eIF2α. MEF were exposed to different concentrations of MeHg for 30 min, and the levels of phosphorylated PERK, total PERK, phosphorylated eIF2 α, total eIF2 α, and β-actin were detected by Western blotting. Cells were treated with 250 mM NaCl for 1 h as positive control. (B) Morphological changes in MEF after challenge with MeHg by pretreatment with or without various concentrations of GSK2606414. Then, the cells were observed by phase contrast microscopy. The cells were then stained with Hoechst 33258 for 15 min. All samples were mounted with Vectashield and observed using fluorescence microscope (Olympus FSX100). Arrows show the apoptotic cells. Scale bar, 50 μm. (C) The percentage of apoptotic (condensed nucleus) cells is represented as the ratio of the total number of cells to the number of apoptotic cells. The data are expressed as the mean±S.E.M. of triplicate cultures run in parallel; $^{**}p<0.01$, $^{***}p<0.001$: significantly different from MeHg-exposed cells without GSK2606414 treatment (ANOVA *post hoc* Bonferroni's test, respectively).

donuclease activity was significantly ameliorated in the MEFs that expressed IRE1α (C931S), but not the IRE1α (C951S) mutants, thereby suggesting that C931 in IRE1α could be a predominant target of MeHg (Figs. 1B, C).

Next, we investigated whether other UPR branches were also modulated by MeHg. Treatment with MeHg stimulated the proteolysis of ATF6 (data not shown) and phosphorylation of PERK and eIF2α (Fig. 2A). A previous study has shown that the IRE1α–XBP1 branch functions as an anti-apoptotic pathway.[18] In contrast, the PERK/ATF6 branches are involved in the induction of cell death.[19] Therefore, these signals may be implicated in the MeHg-induced loss of cell viability. To determine this possibility, we tested the effect of a PERK-specific inhibitor (GSK2606414) on MeHg-induced cell death.[20] Figure 2B shows that stimulation with MeHg triggers a striking change in cell and nuclear morphologies that were characterized by typical apoptotic features including nuclear condensation. Pre-incubation with GSK2606414 for 1 h significantly reduced the number of cells with condensed nucleus, which was a concentration-dependent response to MeHg (Fig. 2C). Apoptotic cell death was abolished by treatment with GSK2606414 in a dose-dependent manner, suggesting that the PERK pathway partially contributes to cell death.

The findings of the present study clearly show that MeHg induces cell death *via* ER stress. Although MeHg stimulates three major UPR sensors, IRE1α endonuclease activity and its downstream signaling are selectively attenuated by the interaction with or modulation of cysteine 931 in the KEN domain of IRE1α. On the other hand, a pharmacological study using GSK2606414 determined that PERK is implicated in MeHg-induced cell death. Here, we report that MeHg disrupts anti-apoptotic signaling based on the IRE1α–XBP1 branch and simultaneously promotes pro-apoptotic signaling *via* the PERK/ATF6 branches. Therefore, the MeHg-induced promotion of pro-apoptotic signaling that is associated with CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) expression may be the predominant mechanism underlying cell death. Several electrophiles that can covalently modify the cysteine residues in proteins may modulate or regulate UPR signaling, particularly IRE1α. Taken together, in conjunction with the MeHg-induced attenuation of the IRE1α component of UPR, the activation of PERK may further sensitize cells to apoptotic death. Furthermore, these findings may be utilized in the development of novel therapeutic approaches for Minamata disease.

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Conflict of Interest The authors declare no conflict of interest.

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