

Demethylation of Methylmercury in Human Neuroblastoma, Glioblastoma and Liver Cells

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The demethylation of methylmercury (MeHg) was investigated in human neuroblastoma, glioblastoma and liver cells. In the three cell lines, MeHg uptake reached a plateau within 24 hr after MeHg exposure while the cellular inorganic Hg levels increased gradually up to 72 hr. Methylviologen, a superoxide anion (O_2^-) generator, was found to be an effective enhancer of demethylation with only a minor effect on lipid peroxidation in all three cases. Fe(II), a hydroxyl radical ($OH\cdot$) enhancer, showed little effect on the reaction in the presence of methylviologen, although lipid peroxidation levels were increased significantly. Furthermore, mannitol, an $OH\cdot$ scavenger, had no effect on inorganic Hg production. These results indicate that MeHg can be converted to inorganic Hg and that O_2^- might predominantly participate in the demethylation reaction in the cells.

Key words — methylmercury, biotransformation, human, superoxide anion

INTRODUCTION

Methylmercury (MeHg) is a ubiquitous environmental pollutant and is widely known to be a neurotoxic compound.^{1,2)} Although MeHg is eas-

ily absorbed from the gastrointestinal tract and distributed to various tissues, the species of Hg found in the target organ may differ from MeHg. In fact, inorganic Hg was found in tissues, including the brain of Minamata victims, examined many years after MeHg exposure.³⁾ However, the contribution of inorganic Hg, formed by biotransformation of MeHg, to neurotoxicity is poorly understood.^{4,5)} Although Takahashi *et al.*³⁾ found that the inorganic Hg in the brain of the above-mentioned victims was in a chemically inert form, they demonstrated that similar inert forms of Hg in rat tissues are not immediately formed after injection of Hg(II) chloride, but tend to increase as time passes. Charleston *et al.*⁶⁾ suggested that inorganic Hg in the thalamus of monkey following subchronic MeHg exposure may be the proximate toxic form of Hg responsible for the changes within the astrocyte and microglial populations. Therefore, inorganic Hg originating from MeHg might be involved in the neurotoxicity before forming chemically inert forms.

Biotransformation of MeHg occurs mainly in the liver, with subsequent accumulation of inorganic Hg in the kidney.⁷⁾ Experimental findings have also found that the reaction occurs in the liver as a result of the interaction of MeHg with superoxide anion (O_2^-).⁸⁾ However, despite both clinical and *in vivo* findings that biotransformation may occur in the brain, the mechanism has yet to be elucidated. We undertook an *in vitro* study using human brain cell lines to confirm the presence of MeHg biotransformation. Second, we examined effects of reactive oxygen modulators on the reaction.

MATERIALS AND METHODS

Chemicals — MeHg chloride, L-cysteine and methylviologen were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). MeHg chloride was used in the experiment without further purification because its purity was confirmed to be > 99.5%. MeHg-cysteine conjugate was freshly prepared before use by dissolving an equivalent amount of L-cysteine into a solution of MeHg chloride in Ca^{2+} , Mg^{2+} -free phosphate-buffered saline [PBS(-)]. 2-Thiobarbituric acid and Cell Counting Kit-8 were obtained from Merck KGaA (Darmstadt, Germany) and Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), respectively. Mannitol, Fe(II) sul-

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fate, Dulbecco's modified Eagle medium (DMEM) and lactate dehydrogenase (LDH)-Cytotoxic Test were products of Wako Pure Chemical Industry, Ltd. (Osaka, Japan). All chemicals used were of the purest grade available.

Cell Cultures—Human neuroblastoma cells SK-N-SH (SK-N-SH) and glioblastoma cells U373MG (U373MG) were obtained from the European Collection of Cell Cultures (Salisbury, U.K.). Human Chang Liver cells (Chang Liver) were kindly donated by Dr. Fusako Usuki, National Institute for Minamata Disease. These cells were maintained in a growth medium consisting of DMEM supplemented with 10% fetal bovine serum (JRH Biosciences Inc., Lenexa, KS, U.S.A.) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Viability Assays—MeHg-induced cytotoxicity was evaluated using the Cell Counting Kit-8 and LDH-Cytotoxic Test.

Cellular Uptake and Demethylation of MeHg—Cells (5.5×10^5 cells/dish) were plated in 100-mm dishes and 24 hr later the medium was replaced to one with or without 1, 2, 3 or 5 μ M MeHg at 37°C. At 24, 48 or 72 hr after MeHg exposure, the cells were washed three times with 6 ml of PBS(–). Total Hg levels were determined by the oxygen combustion-gold amalgamation method⁹⁾ using an atomic absorption detector MD-A (Nihon Instrument Co. Ltd., Osaka, Japan), which was also used to determine inorganic Hg levels as described elsewhere.¹⁰⁾ Protein content of cells was determined by the method of Lowry *et al.*¹¹⁾

Effects of Reactive Oxygen Modulators—Methylviologen, Fe(II) sulfate and mannitol were added 30 min before MeHg exposure, and cells were exposed to MeHg (1 or 2 μ M) for 24 hr. Then total and inorganic Hg levels were determined as above. Lipid peroxidation was estimated from 2-thiobarbituric acid-reactive substance (TBA-RS) levels according to Ohkawa *et al.*¹²⁾

Statistical Analysis—Data are expressed as the mean \pm S.D. The significant difference among the three groups was analyzed by one-way analysis of variance (ANOVA) using Scheffe's multiple comparison posttest when the Bartlett's test proved significant. Differences were considered statistically significant at $p < 0.05$. The significance between the two groups was analyzed by the *t*-test.

RESULTS AND DISCUSSION

Biotransformation of MeHg occurs mainly in the liver, and the mechanism has been elucidated using rat liver slices. Approximately 30% inorganic Hg was found in the rat liver after long-term exposure to MeHg. The levels of inorganic Hg were as low as 5% in the brain.¹³⁾ In an effort to better understand the differences in reaction rates between the brain and the liver, we examined MeHg biotransformation using two brain-derived cell lines, SK-N-SH, U373MG and compared the results to those in Chang Liver cells.

Susceptibility of Three Cell Lines to MeHg

The result of both growth inhibition and viability of cells indicated that SK-N-SH, a type of neuroblastoma cell, is most susceptible to MeHg among the three cell lines tested (data not shown). Based on these results, we chose 1 μ M of MeHg for SK-N-SH and up to 5 μ M for U373MG and Chang Liver in the subsequent experiment.

Accumulation and Degradation of MeHg

Figure 1A–1C shows the time courses of Hg accumulation after MeHg exposure in the three cell lines. In the three cell lines, cellular Hg levels reached a plateau within 24 hr after MeHg exposure. Interestingly, the Hg accumulation after 1 μ M of MeHg exposure was highest in SK-N-SH cells. On the contrary, cellular inorganic Hg levels increased gradually up to 72 hr in each cell line (Fig. 1D–1F). These results may suggest that biotransformation of MeHg generally takes place in these human cell lines. In U373MG and Chang Liver cells, the accumulation of MeHg and production of inorganic Hg increased in a dose-dependent manner. After 1 μ M of MeHg exposure for 48 or 72 hr, the portion of inorganic Hg in Chang Liver cells was 2-times higher than that in SK-N-SH cells, indicating a high activity of MeHg demethylation in Chang Liver cells. These results reflect *in vivo* experimental findings that the portion of inorganic Hg is higher in the liver than the brain.^{13, 14)}

Our results showed that SK-N-SH cells are the most sensitive to MeHg cytotoxicity among the three cell lines tested but the biotransformation activity is low. Magos *et al.*¹⁵⁾ have reported that the slow dealkylation cannot be the cause of neurotoxicity in rats following acute alkylmercurial exposure. Consequently, it is likely that the contribution of MeHg biotransformation to the cytotoxicity

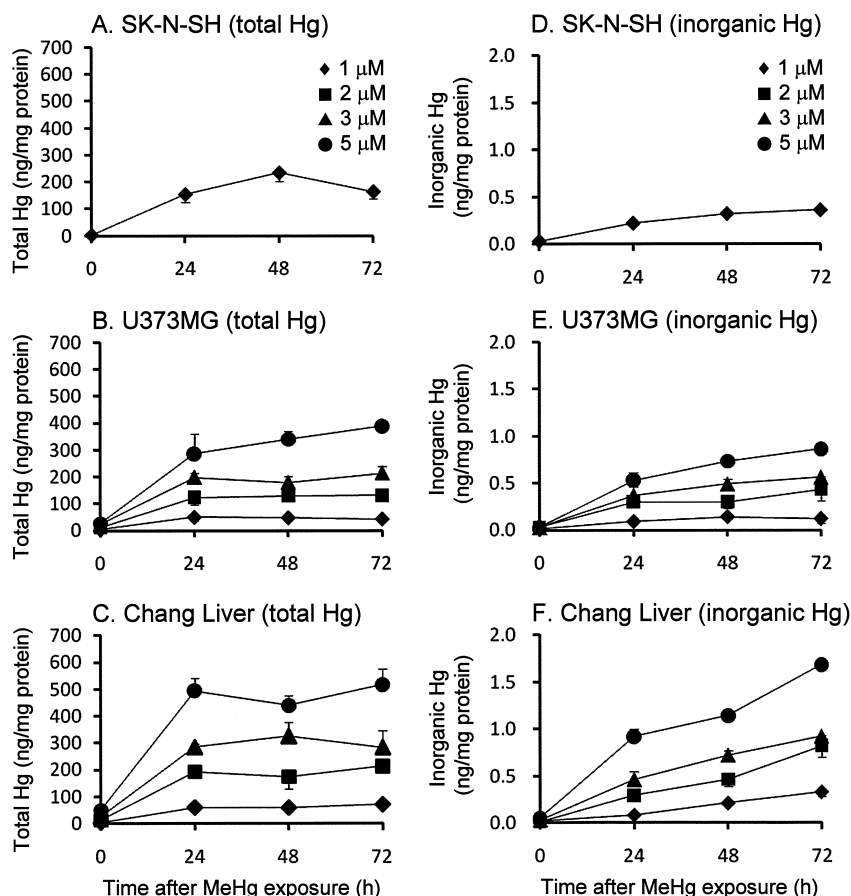


Fig. 1. Time-course of Total Hg Accumulation and Inorganic Hg Production in Three Cell Lines after MeHg Exposure
Values represent the mean \pm S.D. ($n = 3-10$).

is also low in the present study. Also, since inorganic Hg, in contrast to MeHg, can bind to metallothionein, which works to detoxify various heavy metals or can be converted to an inert form,³⁾ the biotransformation of MeHg may be interpreted as a part of biological processes to lower the toxicity of MeHg.

Effects of Reactive Oxygen Modulators

Yasutake and Hirayama⁸⁾ suggested that the demethylation of MeHg by the liver slice would proceed with the aid of O_2^- produced in the electron transfer system at the hydrophobic mitochondrial inner membrane. Recently, Shapiro *et al.*¹⁶⁾ have demonstrated using a primary culture of rat astrocytes that demethylation of MeHg was enhanced under oxidative stress. To examine the involvement of reactive oxygen species (ROS) in reactions in human cell lines, we investigated the effects of reactive oxygen modulators.

Addition of methylviologen, an O_2^- generator, significantly increased the portion of inorganic Hg,

compared with the MeHg alone group (Fig. 2). This implies the participation of O_2^- and/or its metabolites in MeHg degradation. The high portion of inorganic Hg detected in the methylviologen-treated U373MG cells might also imply that the O_2^- production systems in the cells are highly sensitive to the reagent. Since hydroxyl radical ($OH\cdot$) has been shown to be effective at cleaving the C-Hg bond in MeHg,¹⁷⁾ we also examined the effect of $OH\cdot$ on inorganic Hg formation. Addition of Fe(II), an $OH\cdot$ enhancer, significantly increased the TBA-RS level in the cells (Fig. 2), and therefore the O_2^- produced here would be converted to $OH\cdot$ by the catalytic action of Fe(II). However, addition of Fe(II) only minimally impacted the methylviologen-induced inorganic Hg production in the cells, suggesting that the contribution of $OH\cdot$ to the reaction is much lower than O_2^- . The fact that mannitol, an $OH\cdot$ scavenger, did not affect the reaction at all (Fig. 3) confirms that there is a very small contribution of $OH\cdot$ to MeHg degradation. Yasutake and Hirayama⁸⁾ showed that MeHg was effectively converted to inorganic Hg by

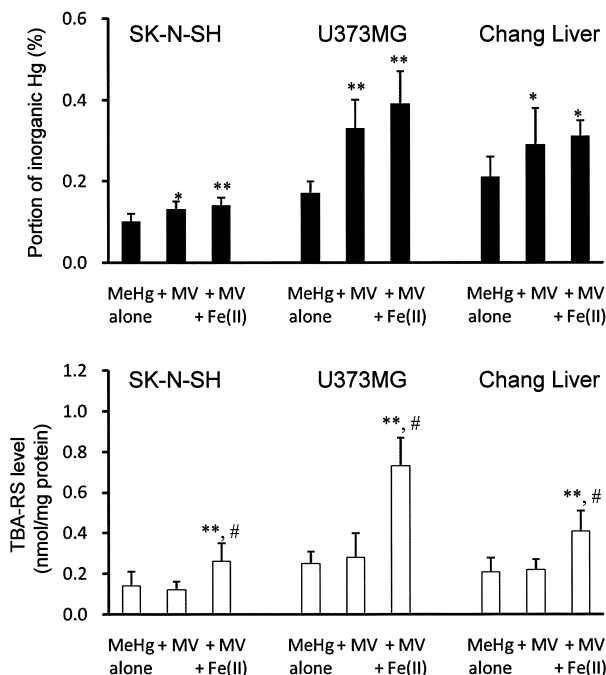


Fig. 2. Effects of Methylviologen and Ferrous Ion on Inorganic Hg Formation and Lipid Peroxidation in Three Cell Lines

After pretreatment of methylviologen (MV, 100 μ M) and Fe(II) sulfate [Fe(II), 10 μ M] for 30 min, cells were exposed to MeHg (1 or 2 μ M) for 24 hr. The amount of total- and inorganic Hg formed was determined. The levels of TBA-RS were also determined. Values represent the mean \pm S.D. ($n = 6$). **Significantly different from MeHg alone ($p < 0.05$, $p < 0.01$). #Significantly different from MV pretreatment ($p < 0.01$).

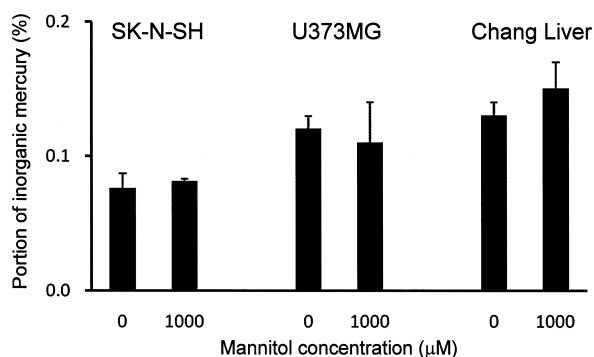


Fig. 3. Effect of Mannitol on Inorganic Hg Formation in Three Cell Lines

After pretreatment of mannitol for 30 min, cells were exposed to MeHg (1 or 2 μ M) for 24 hr. The amount of total- and inorganic Hg formed was determined. Values represent the mean \pm S.D. ($n = 3$).

O_2^- in organic solvents, while in the aqueous solution, MeHg was quite stable. Thus, the presence of O_2^- would be sufficient for the reaction if it functions in a hydrophobic environment such as the mitochondrial membrane. Accordingly, we concluded

that the involvement of OH^\bullet to MeHg demethylation is minimal in all three cell lines.

In the present study, we investigated MeHg biotransformation using human cell lines, and could reproduce the reaction previously reported *in vivo*. Our findings suggested that MeHg biotransformation takes place in the human brain and that except for OH^\bullet , ROS are predominantly involved in the reaction, with O_2^- being a likely candidate. MeHg biotransformation in human cell lines requires further investigation to delineate the mechanisms involved.

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