INTRODUCTION

Metallothionein (MT) is a cysteine-rich protein found in various animal tissues as 4 isomers, MT-I, II, III and IV, to function as a scavenger of heavy metals as well as a pool for essential metals. Various heavy metals including inorganic mercury (Hg(II)) are well documented to induce metallothionein (MT) in tissues of injected animals. Although Hg(0) and MeHg are considered to be inert in terms of directly inducing MT, MT can be induced by them after *in vivo* conversion to Hg(II) in an animal body. In the present study we examined accumulations of inorganic mercury and MT inductions in mouse tissues (brain, liver and kidney) up to 72 hr after treatment by one of three mercury compounds of sub-lethal doses. Exposure to mercury compounds caused significant mercury accumulations in mouse tissues examined, except for the Hg(II)-treated mouse brain. Although MeHg caused the highest total mercury accumulation in all tissues among mercury compounds, the rates of inorganic mercury were less than 10% through the experimental period. MT inductions that depended on the inorganic mercury accumulation were observed in kidney and brain. However, MT induction in the liver could not be accounted for by the inorganic mercury accumulation, but by plasma IL6 levels, marked elevation of which was observed in Hg(II) or MeHg-treated mouse. The present study demonstrated that MT was induced in mouse tissues after each of three mercury compounds, Hg(0), Hg(II) and MeHg, but the induction processes were different among tissues. The induction would occur directly through accumulation of inorganic mercury in brain and kidney, whereas the hepatic MT might be induced secondarily through mercury-induced elevation in the plasma cytokines, rather than through mercury accumulation in the tissue.

**Key words:** Mercury vapor, Inorganic mercury, Methylmercury, Metallothionein, IL6

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**ABSTRACT** — Among the naturally occurring three mercury species, metallic mercury (Hg(0)), inorganic mercury (Hg(II)) and methylmercury (MeHg), Hg(II) is well documented to induce metallothionein (MT) in tissues of injected animals. Although Hg(0) and MeHg are considered to be inert in terms of directly inducing MT, MT can be induced by them after *in vivo* conversion to Hg(II) in an animal body. In the present study we examined accumulations of inorganic mercury and MT inductions in mouse tissues (brain, liver and kidney) up to 72 hr after treatment by one of three mercury compounds of sub-lethal doses. Exposure to mercury compounds caused significant mercury accumulations in mouse tissues examined, except for the Hg(II)-treated mouse brain. Although MeHg caused the highest total mercury accumulation in all tissues among mercury compounds, the rates of inorganic mercury were less than 10% through the experimental period. MT inductions that depended on the inorganic mercury accumulation were observed in kidney and brain. However, MT induction in the liver could not be accounted for by the inorganic mercury accumulation, but by plasma IL6 levels, marked elevation of which was observed in Hg(II) or MeHg-treated mouse. The present study demonstrated that MT was induced in mouse tissues after each of three mercury compounds, Hg(0), Hg(II) and MeHg, but the induction processes were different among tissues. The induction would occur directly through accumulation of inorganic mercury in brain and kidney, whereas the hepatic MT might be induced secondarily through mercury-induced elevation in the plasma cytokines, rather than through mercury accumulation in the tissue.

**Key words:** Mercury vapor, Inorganic mercury, Methylmercury, Metallothionein, IL6

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to Hg(II) in animal tissues, and an involvement of reactive oxygen species in the reaction is suggested (Hirayama and Yasutake, 1999; Yasutake and Hirayama, 2001). Although Hg(0) and MeHg are considered to be inert in terms of directly inducing tissue MT, they can be changed to Hg(II), a typical MT-inducing metal, in animal tissues. Accordingly, Hg(0) and MeHg also possibly induce tissue MT after in vivo conversion to Hg(II). In the present study we examined accumulations of inorganic mercury and MT inductions in brain, kidney and liver of mice after exposure to sub-lethal doses of Hg(0), Hg(II) and MeHg, and considered the difference in the MT induction processes. Since the cerebellum is known to be more susceptible to MeHg toxicity than the cerebrum (Nakamura et al., 2007, 2011), responses of these two tissues were separately examined here.

MATERIALS AND METHODS

Animals and treatment

Male C57BL/6N mice (8 weeks of age) were obtained from Kyudo Co., Ltd. (Kumamoto, Japan), and maintained at 23 ± 2°C, 55% humidity, and allowed to free access to laboratory chow and water. Fifteen mice in each group were treated by Hg0 vapor (8.3 mg/m3 for 30 min), HgCl2 (2.5 μmol/kg, ip) or MeHgCl (80 μmol/kg, po). At 6, 24 and 72 hr after the mercury treatment, 5 mice in each group were sacrificed under ether anesthesia to obtain cerebrum, cerebellum, liver and kidney samples for mercury, MT and RNA determinations. Additionally, lipid peroxide and GSH levels in the tissues were determined for the 24-hr group. Furthermore, blood samples were collected from the 6-hr group to obtain plasma samples for cytokine assays. The tissue samples for GSH analysis were immediately frozen in liquid nitrogen, and stored at -80°C.

Mercury, MT and lipid peroxide levels

For MT, Hg and lipid peroxide analyses, the tissue samples were homogenized (10% w/v) in N2-saturated ice-cold 5% perchloric acid containing 1 mM EDTA, and stored at -80°C until analysis. The samples for RNA preparation were immediately frozen in liquid nitrogen, and stored at -80°C.

For each tissue, an aliquot of the homogenate was subjected to MT assay according to the procedure of Nagatomo et al. (1987) with a slight modification using non-radioactive HgCl2 (Yasutake et al., 1998). Briefly, the homogenate was treated successively with diethylmalate and 10 mM CdCl2, then heated at 95°C for 5 min to precipitate high-molecular weight proteins. Following cooling and centrifugation, the supernatant was successively treated with 5 mM HgCl2, 1 mM ovalbumin, and 12.5% TCA. After centrifugation, the supernatant was filtered through a membrane of 0.22-μm pore diameter (Ultrafree C3, Millipore - Japan, Tokyo, Japan) to provide Hg-MT samples. The total MT levels were determined by analyzing Hg concentration in the final filtrate as described above, and expressed as amount of mercury bound to the thionein molecules. To estimate the lipid peroxide level, an aliquot (100 μl) of the homogenate was immediately diluted (10% v/v) with ice-cold 1.15% KCl containing 0.01% butylhydroxytoluene to prevent auto-oxidation, and the 2-thiobarbituric acid reactive substance (TBARS) level was measured according to Ohkawa et al. (1979).

GSH analysis

For total GSH analysis, c.a. 0.1 g portions of liver and kidney were immediately homogenized in ice-cold 4% perchloric acid containing 1 mM EDTA (1 ml). After centrifuge (12,000 rpm x 1 min), the supernatant fraction was used in GSH analysis by the enzymatic recycling method according to Tietze (1969).

Quantitative reverse transcription (RT)-PCR

Total RNA was isolated from the mouse tissue samples by the RNaseasy mini kit (QIAGEN K.K., Tokyo, Japan) and reverse transcription (RT) reactions were done using ExScript RT reagent Kit (Takara BIO INC., Shiga, Japan) according to the manufacturer’s instructions. External standard, consisting of serial dilutions of mouse MT-I, MT-II, MT-III and serum amyloid A1 (Saa1) cDNA (105, 104, and 103 copies), were constructed by RT-PCR. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized. To evaluate the mouse MT-I, MT-II, MT-III and Saa1 and GAPDH mRNA copies, the upstream and downstream primer sequences were listed in Table 1.

Real-time RT-PCR was done using a LightCycler (Roche Diagnostics, Tokyo, Japan) as follows. The real-time RT-PCR reaction was performed in the LightCy-
cler glass capillaries in a total volume of 20 μl: 1 μl of RT reaction was added to 10 μl of 2 x SYBR Premix Ex Taq, 0.4 μl of 10 μM of each primer and DEPC-H2O to the final volume. Conditions of real-time RT-PCR were as follows: 10 sec at 95°C (initial activation), and 40 cycles of amplification for 5 sec at 95°C (denaturation) and 20 sec at 60°C (annealing and extension). All heating and cooling steps were performed with a slope of 20°C/sec. A single fluorescence reading at 530 nm was obtained for each sample at the extension step.

The crossing point (CP) values of these standards were used to generate an external standard curve providing accurate quantification. The ratio of mouse MT-I, MT-II, MT-III and Saa1 mRNA copies to mouse GAPDH mRNA copies was estimated.

Cytokine assays
Interleukin-6 and TNFα levels in the plasma samples obtained 6 hr after mercury treatment were determined using a Mouse IL-6 Chemiluminescent ELISA (Pierce Protein Research Products, Rockford, IL, USA) and Mouse TNFα ELISA KIT (Shibayagi Co., Ltd., Gunma, Japan) according to the manufacturer’s protocols.

Table 1. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt1</td>
<td>Forward</td>
<td>5' - CACCAGATCTCGGAATGGAC - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' - GAGGCTGCACTTGCAGTTCTTG - 3'</td>
</tr>
<tr>
<td>Mt2</td>
<td>Forward</td>
<td>5' - CGCTGCAAATGCAAACAAT - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' - CTGCACTTTGTGGAAGCCCTCT - 3'</td>
</tr>
<tr>
<td>Mt3</td>
<td>Forward</td>
<td>5' - TGCAAATGCAACTGCAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' - CCAGGGACACCCACCATTTAC - 3'</td>
</tr>
<tr>
<td>Saa1</td>
<td>Forward</td>
<td>5' - GAGTCTGGGCTGCTGAGAA - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' - ACTGCGGCCATGTCTGTTG - 3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>5' - AAAATGGAAGGCTGCTGTGTTG - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5' - TGAAGGGGTCGGTGGATGG - 3'</td>
</tr>
</tbody>
</table>

Table 2. Total mercury concentrations (μg/g) in tissues of MeHg-exposed mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr (Control)</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.0128 ± 0.0039</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.0145 ± 0.0011</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.130 ± 0.006</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0236 ± 0.0062</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D. obtained from five mice. Numbers in parentheses show mean rate (%) of inorganic mercury.

Results in the behavioral tests were expressed as mean ± S.D., and differences from control mice were analyzed by Student’s t-test. P value < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Mercury accumulation
To observe clear effects by three mercury compounds on tissue MT, about half of the lethal doses were employed in the present study. The present dose levels of HgCl2 (2.5 μmol/kg, ip) and MeHg (80 μmol/kg, po) are enough to cause a significant renal dysfunction 24 hr after the treatment (Yasutake et al., 1990). Since a part of mice were found to die within 3 days after Hg(0) exposure of 8.3 mg Hg/m3 for 1 hr, exposure time for 30 min was employed here. Through the treatment of each of three mercury compounds, significant accumulations of inorganic mercury were observed in mouse tissues except for the brain in the HgCl2-treated group (Fig. 1). This indicated the effective role of the blood-brain barrier against Hg2+ penetration. On the other hand, Hg(0) and MeHg
in the blood circulation easily enter the brain by diffusion (WHO, 1991) and via the amino acid transporter (Hirayama, 1985; Aschner, 1989), respectively. The kidney showed the highest accumulation among the tissues examined by all mercury compounds. MeHg caused the highest total mercury accumulation in all tissues among three mercury compounds; the highest accumulation was shown in the kidney at 6 hr (Table 2). However, its rate of inorganic mercury was as low as 0.417%, and the rates were less than 10% through the experimental period in all tissues. Since the *in vivo* conversion of MeHg to the inorganic form proceeded gradually (Hirayama and Yasutake, 1999; Yasutake and Hirayama, 2001), the tissue accumulation of inorganic mercury increased in a time-dependent manner in all the tissues of MeHg-treated mice (Fig. 1C). On the other hand, tissue mercury levels showed monotonic decreases in Hg(0)- and HgCl2-treated mice (Figs. 1A and B), indicating very quick uptake of these mercury compounds.

**MT mRNA**

Induction rates of MT mRNAs in brain, kidney and liver 6 hr after treatment by mercury compounds were examined using quantitative RT-PCR. The induction rates in the brain were rather small. Especially in the cerebrum, the rates were at most 1.3 times of the control for MT-II mRNA (Fig. 2A). On the other hand, three mercury compounds induced MT-I and II mRNAs in the cerebellum up to 2.3 times (Fig. 2B), suggesting that the cerebellum is more susceptible than the cerebrum to challenges by three mercury compounds. However, mercury exposure caused significant increases in MT mRNAs in kidney and liver. Kidney MT-I and II mRNAs increased by 18 to 25 times 6 hr after Hg(0) and HgCl2 treatment, though the induction rate by MeHg was quite small (Fig. 2C), probably due to a low accumulation of inorganic mercury at that time. On the other hand, the induction feature in the liver was quite different from in the kidney. Hg(II) injection caused 13.1 and 12.4 times the inductions at 6 hr for MT-I and II mRNAs in the liver, respectively. However, Hg(0) exposure caused only 1.5 to 2.5 times the inductions in the liver MT-I and II mRNA (Fig. 2D), though the tissue accumulation of mercury at that time was similar to that in Hg(II)-treated mice (Figs. 1A and B). Interestingly, MeHg, which showed the lowest induction rate in the kidney MTs, caused the highest induction in the liver MTs among mercury compounds (Fig. 2D). The induction rates in the liver were 18 and 41 times those for MT-I and II mRNAs, respectively.

**Total MT level**

Increases of the tissue total MT levels after exposure to...
three mercury compounds were mostly compatible with the alterations observed in the RNA levels. The increases were evident in liver and kidney, but very slight in cerebrum and cerebellum (Fig. 3). Despite significant increases in the cerebellum mRNAs as mentioned above (Fig. 2B), increases in MT were very little, suggesting that RNA inductions up to 2.3 times might not be sufficient to cause significant MT increases. Hg(0) exposure caused a significant increase in the renal MT levels by 6.4 times 6 hr after exposure, and showed a time-dependent decrease with the reduction in mercury accumulation (Figs. 1A and 3A). However, alteration in the hepatic MT by Hg(0) was rather small. Hg(II) also caused an initial increase and time-dependent decrease in the renal MT levels as observed in Hg(0) exposure. As different from Hg(0), the Hg(II) injection caused a significant increase in the hepatic MT by 8 times at 6 hr (Fig. 3B), though the tissue mercury accumulation levels were similar between Hg(0)- and Hg(II)-treated mice (Fig. 3A and B). MeHg injection caused significant increases of the tissue MT in kidney and liver (Fig. 3C). The kidney level showed a time-dependent increase as observed in the inorganic mercury accumulation (Fig. 1C). On the other hand, the liver MT drastically increased by about 60 times from 6 hr to 24 hr, then declined thereafter (Fig. 3C).

Fig. 2. RNA expression levels of MT isomers in (A) cerebrum, (B) cerebellum, (C) kidney and (D) liver of mice exposed to mercury compounds. Expression levels were determined by real-time RT-PCR, and shown as levels relative to the control mice. Each value represents mean ± S.D. obtained from four mice. Significant differences from control mice were shown by * (p < 0.05) and ** (p < 0.01).
Involvement of oxidative stress and cytokines

Since MT is well known to be induced by oxidative stress and some cytokines, such as IL6 and TNFα, their involvements were examined 24 hr after mercury exposure. The extent of oxidative stress was estimated by lipid peroxide levels and alterations in tissue GSH levels, since GSH was induced by oxidative impact as MT. Lipid peroxide levels estimated by TBA-RS assay in liver and kidney were quite stable even after treatment of mercury compounds (data not shown). On the other hand, the renal GSH levels were significantly induced by Hg(0) and Hg(II), whereas the liver levels were lowered by these two mercury compounds (Fig. 4). Previously we found in MeHg-treated mice that the γ-glutamylcysteine synthetase activity in the kidney was induced, while the turnover rate of GSH was increased in the liver (Yasutake and Hirayama, 1994). Similar effects might have occurred here in Hg(0)- and Hg(II)-treated mice. The brain GSH levels were not at all affected by each mercury treatment (data not shown).

It should be noted that IL6 levels in mouse plasma were significantly elevated after Hg(II) and MeHg exposure (Fig. 5), but Hg(0) exposure caused no alteration.
Despite comparable mercury accumulations in the livers of Hg(0)- and Hg(II)-treated mice, significant MT induction was observed only in the Hg(II) group as mentioned above. The elevated IL6 caused by Hg(II) injection would stimulate MT induction in the liver, since IL6 receptor is rich in mouse liver (Raynes et al., 1991). A slight MT induction in Hg(0)-exposed mouse liver suggested that inducing activity by inorganic Hg itself in the liver of this strain mouse might be quite small. Significant MT induction in MeHg-treated mouse liver would also be due to the increased plasma IL6. However, alteration of another possible cytokine TNFα could not be detected in the mice plasma (data not shown). Serum amyloid A1 (Saa1) in the liver was also reported to be induced by IL6 (Raynes et al., 1991). Since expression levels of the hepatic Saa1 were markedly enhanced by the Hg(II) and MeHg groups, but slightly by Hg(0) (Fig. 6) as observed in the MT inductions, the elevation of IL6 would account for the MT inductions in the liver observed here.

The present study demonstrated that MT was induced in mouse tissues after three mercury compounds, Hg(0), Hg(II) and MeHg, but the induction processes were different among tissues. The induction would occur directly through accumulation of inorganic mercury in brain and kidney, whereas the hepatic MT might be induced secondarily through mercury-induced elevation in the plasma cytokines, rather than through mercury accumulation in the tissue.

![Plasma IL6 levels in mice after exposure to Hg(0), HgCl2, and MeHg. Each value represents mean ± S.D. obtained from five mice. Significant differences from control mice were shown by * (p < 0.05) and ** (p < 0.01).](image1)

![Expression levels of Saa1 in mouse liver exposed to mercury compounds. Each value represents mean ± S.D. obtained from five mice. Significant differences from control mice were shown by * (p < 0.05) and ** (p < 0.01).](image2)

ACKNOWLEDGMENT

The authors wish to thank Ms. M. Ogata for her technical assistance in the Hg analysis and animal treatment. The experimental protocol was approved by the Ethics Committee for Research on Animals in the National Institute for Minamata Disease.

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