S-Mercuration of cellular proteins by methylmercury and its toxicological implications

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ABSTRACT — The accumulation of methylmercury (MeHg) through the daily consumption of large predatory fish poses potential health risks. MeHg has been found to cause Minamata disease, but the full nature of MeHg toxicity remains unclear. Because of its chemical properties, MeHg covalently binds to cellular proteins through their reactive thiols, referred to as S-mercuration, resulting in the formation of protein adducts. In this review, we summarize how the S-mercuration of cellular proteins could be involved in the major mechanisms that have been suggested to underlie MeHg toxicity. Additionally, we introduce our attempts to identify cases of S-mercuration for the research to reveal the true nature of MeHg toxicity.

Key words: Methylmercury, Cysteine, Thiol, Covalent binding, Protein

INTRODUCTION

Many humans have unknowingly accumulated methylmercury (MeHg), which is an environmental electrophile, in the body. Mercury is widespread and persistent in the environment, and its use in many products and the emission of mercury from combustion processes have resulted in chronic environmental exposure around the world. Once in the environment, mercury can be converted into MeHg, which can bioaccumulate and biomagnify through the food chain. There have been concerns about health effects, including damage to the central nervous system (Chang, 1990; Shimai and Satoh, 1985), caused by the accumulation of MeHg through the daily consumption of large predatory fish. This does not occur under conditions typical for most people, however, in a particular incident, MeHg was released from a chemical plant and was bioaccumulated by fish and shellfish in Minamata Bay. This resulted in people consuming fish and shellfish from that bay experiencing mercury poisoning, which has since been referred to as Minamata disease. Despite it having been more than half a century since Minamata disease was discovered, the molecular mechanisms underlying the toxic effects of MeHg are still poorly understood, and more research is required to improve our understanding of the mechanisms involved.

There are about 214,000 cysteine residues encoded in the human genome (Jones, 2008). Cysteine residues are found at the active sites of many enzymes, and they regulate the functions and maintain the conformations of the proteins. Most cysteine residues are buried within the molecule and form disulfide bonds to maintain the structure of the protein. They can, alternatively, serve as ligands for zinc ions (Zn²⁺). Between 10% and 20% of the thiol groups in proteins are dissociated, as thiolate anions, under physiological conditions because their pKₐ value is low, and this means that they can be potential targets for electrophiles that have low electron densities. For this reason, exogenous electrophiles could cause accidental chemical modifications of proteins (Kumagai and Sumi, 2007; Kumagai et al., 2012). MeHg has a particularly high association constant for thiols (10⁻¹⁵⁻¹⁰¹⁶; Table 1) (Simpson, 1961), and its chemical properties allow MeHg to become covalently bounded to protein thiols in the body, a modification that is termed “S-mercuration.” The cellular response to electrophiles is typically biphasic, with the activation of protective signaling pathways occurring at low concentrations and cell death occurring at higher concentrations (Levonen et al., 2014). Unintentional S-mercuration disrupts biological homeostasis, and this is thought to be associated, at least in part, with the onset of MeHg toxicity (Fig. 1).
As described above, it has been well known for a long time that MeHg causes the S-mercuration of cellular proteins. However, the toxicological effects of S-mercuration have been assessed in detail in very few publications. Here, we wish to assess what has become clear about the relationship between the S-mercuration of proteins and the onset of toxic effects. To achieve this, we will examine the types of S-mercured proteins that it has been suggested could be involved in the main mechanisms that underlie MeHg toxicity. We will also introduce our attempts to identify cases of S-mercuration.

**Table 1. Association constants for MeHg and various ligands**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Association constant (logarithm)</th>
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<tbody>
<tr>
<td>SH (Cysteine)</td>
<td>15.7</td>
</tr>
<tr>
<td>NH₂ (Histidine)</td>
<td>8.8</td>
</tr>
<tr>
<td>NH₃</td>
<td>8.4</td>
</tr>
<tr>
<td>Imidazole</td>
<td>7.3</td>
</tr>
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**MAJOR MECHANISMS UNDERLYING METHYLMERCURY TOXICITY**

To determine the major mechanisms that have been suggested to underlie MeHg toxicity, we searched the literature published up to May 2014 for the terms “methylmercury”, “mechanism”, and “review” using PubMed. A total of 30 publications matching the search terms were identified, and nine of them were found to include a discussion of the mechanisms involved in MeHg toxicity. We found that five major mechanisms underlying MeHg toxicity have been identified, and these are summarized in Table 2, along with the factors associated with those mechanisms that were mentioned in the articles. The main mechanisms that have been identified involve oxidative stress and changes in the intracellular concentrations of calcium ions (Ca²⁺). An example of another type of impaired signaling that was suggested to be caused by MeHg was the inhibition of glutamate uptake. The inhibition of protein synthesis and the disruption of microtubules as the well-known mechanisms were also extracted. According to the textbook “Toxicological Effects of Methylmercury”, published by the US National Research Council’s Board on Environmental Studies and Toxicology (Committee on the Toxicological Effects of Methylmercury, 2000), mitochondrial changes, the induction of lipid peroxidation, microtubule disruption, and disrupted protein synthesis have been proposed as being possible mechanisms for MeHg toxicity. These mechanisms are similar to those found from the published literature identified in our search. Protein inhibition was the most common factor that was mentioned in the articles that we inspected.

**THE RELATIONSHIP BETWEEN CELLULAR DYSFUNCTION BY METHYLMERCURY AND S-MERCURATION OF CELLULAR PROTEINS**

We attempted to assess the mechanisms through which the S-mercuration of cellular proteins could be involved in the cellular dysfunction described above, using even publications that had not actually been referred to in the nine most relevant publications that we had identified. The cellular proteins that can potentially be S-mercured are summarized in Table 2, and they are shown schematically in Fig. 2.

**Oxidative stress and S-mercuration**

Oxidative stress is a disturbance in the redox status (the balance between oxidants and antioxidants) in the body. MeHg increases the production of reactive oxygen species (ROS) and the degree to which lipids are peroxidized (Ali et al., 1992; Dreiem and Seegal, 2007; Garg and Chang, 2006; LeBel et al., 1990, 1992; Lee et al., 2009; Mori et al., 2007; Yin et al., 2007; Yonaha et al., 1983). Antioxidant enzymes such as superoxide dismutase (SOD), cat-
### Table 2. Mechanisms underlying MeHg toxicity and factors that have been suggested to be involved in the toxic responses in the eligible publications, and the cellular proteins that are potentially S-mercurated

<table>
<thead>
<tr>
<th>Mechanism and features</th>
<th>Factors</th>
<th>Articles</th>
<th>Potential S-mercurated proteins</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1. Oxidative stress (increased reactive oxygen species (ROS); decreased endogenous antioxidant defense) | ● Decrease in intracellular reduced glutathione (GSH) caused by the inhibition of cysteine transport and the formation of a covalently bounded GS–MeHg complex  
● Alterations in antioxidative enzymes (e.g., glutathione peroxidase (GPx) and thioredoxin reductase (TrxR))  
● Changes in intracellular concentrations of Ca^{2+} | Fonnum and Lock, 2004; Patel and Reynolds, 2013; Shanker and Aschner, 2001 | Mn-SOD | Kumagai et al., 1997; Shinyashiki et al., 1996 |
| 2. Changes in intracellular concentrations of Ca^{2+} | ● Increase in plasma membrane permeability (Inhibition of Ca^{2+} channels and Na^{+}/K^{+}-ATPase; ROS or activation of cellular lipases)  
● Release from intracellular Ca^{2+} pool (including inhibition of muscarinic receptors)  
● Increase in the intracellular concentration of an endogenous polyvalent cation (e.g., Zn^{2+}) because of its release from soluble proteins | Atchison and Hare, 1994; Denny and Atchison, 1996; Fonnum and Lock, 2004 | Ca^{2+} -ATPase | Freitas et al., 1996 |
| 3. Inhibition of glutamate uptake | ● Inhibition of glutamate transporters (EAAT-1/GLAST and EAAT-2/GLT-1) | Aschner, 2000; Aschner et al., 2000; Fonnum and Lock, 2004 | EAAT-1 (GLAST) (?) | Aschner et al., 2000 |
| 4. Inhibition of protein synthesis | ● Direct interaction with some parts of the protein synthetic machinery (including inhibition of the aminoacyl-tRNA synthetase enzymes) | Atchison and Hare, 1994; Clarkson, 1987; Miura and Imura, 1987 | Aminoacyl-tRNA synthetase (?) | Cheung and Verity, 1985 |
| 5. Disruption of microtubules | ● Reaction with SH groups in tubulin | Clarkson, 1987; Miura and Imura, 1987 | Tubulin | Vogel et al., 1985, 1989 |

* Tentative
alase, and glutathione peroxidase (GPx) play important roles in scavenging oxidants and preventing cell injury. The brain is particularly sensitive to oxidative damage because it contains many oxidizable substrates, such as lipids (Halliwell, 1992), with low SOD and GPx activities (Mori et al., 2007). We summarize the effect MeHg has on these antioxidant enzymes below.

Yee and Choi (1994) found a marked decrease in the total SOD activity in mouse brains when the mice had been treated with MeHg for a prolonged period (at 2.5 mg/kg/day for 7 days). The authors didn’t determine which isozyme was involved in the enzyme loss, but eukaryotic cells contain two types of SODs, copper and zinc SOD (Cu,Zn-SOD) and manganese SOD (Mn-SOD). We later found that Mn-SOD is susceptible to being modified by MeHg and that Cu,Zn-SOD is not (Shinyashiki et al., 1996). Exposing mice to MeHg at a dose of 10 mg/kg only once resulted in a time-dependent decrease in Mn-SOD activity but had little effect on Cu,Zn-SOD activity in the brain. Mn-SOD is found in mitochondria, in which more than 90% of the oxygen provided to a cell is consumed and in which ROS are generated. The importance of Mn-SOD in protecting against MeHg toxicity was reported by Naganuma et al. (1998). They found that the sensitivity of HeLa cells to MeHg was decreased by the overexpression of Mn-SOD, suggesting that the formation of superoxide anions in the mitochondria might be involved in the mechanism(s) involved in the cytotoxicity of MeHg. We have attempted to examine the mechanism involved in the relationship between Mn-SOD and MeHg (Kumagai et al., 1997; Shinyashiki et al., 1996). Levels of mRNA and protein synthesis for Mn-SOD were unaffected by MeHg administration. The direct effect of MeHg on the Mn-SOD and Cu,Zn-SOD activities was further examined using purified enzyme preparations. MeHg caused a facile reduction in Mn-SOD activity but not that of Cu,Zn-SOD. A combination of isoelectric focusing agarose gel electrophoresis and synchrotron radiation X-ray fluorescence analysis revealed that Mn-SOD rather than Cu,Zn-SOD underwent modification by MeHg. The SH groups in both Cys140 and Cys196 in Mn-SOD are exposed at the subunit surface. Experiments involving thiol titration with Ellman’s reagent and the purification of digested fragments of human Mn-SOD using thiol-Sepharose column chromatography, each followed by amino acid sequence determination, indicated that Cys196, but not Cys140, is the reactive sulfhydryl (Matsuda et al., 1990). Therefore, oxidative stress caused
by MeHg is at least partly due to the S-mercuration of Mn-SOD, presumably through Cys196.

In addition to this SOD-related research, it has been found in a number of studies that decreased GPx activity in the brains was caused by the mice being given MeHg at a concentration of 40 mg/L in drinking water for several weeks (Carvalho et al., 2007; Farina et al., 2003b; Franco et al., 2009; Glaser et al., 2010). Stringari et al. (2008) reported that exposing pregnant mice to MeHg at concentrations of 1, 3, and 10 mg/L in drinking water induced a dose-dependent and long-lasting inhibitory effect on the occurrence of cerebral antioxidant enzymes, such as GPx, which is a developmental phenomenon, in their pups. There have also been many reports of MeHg causing decreased GPx activity in vitro (Farina et al., 2009; Hirota et al., 1980; Kromidas et al., 1990). Farina et al. (2009) reported that the overexpression of GPx1, which is the most abundant version of GPx, prevented MeHg-induced neuronal death, indicating that GPx1 is an important molecular target that is involved in MeHg-induced neurotoxicity. They didn’t determine how GPx1 was inhibited, but suggested that selenol groups (which have lower pKa values than do thiol groups) in the enzyme were involved. However, thiols are much more abundant than selenols (Farina et al., 2011). In fact, thiol groups can be found in both low-molecular-weight (mainly reduced glutathione, GSH) and high-molecular-weight proteins, whereas selenol groups are found only in a restricted group of selenoproteins (Araia and Shiraiwa, 2009; Lobanov et al., 2009). Consequently, MeHg is expected to be found primarily in thiol-containing proteins (and much less in selenol-containing proteins) in edible fish muscles, which are the most important environmental sources of MeHg to humans (Farina et al., 2011). MeHg has also been found to cause the inactivation of thioredoxin reductase (another selenoprotein; TrxR) both in vivo and in vitro (Branco et al., 2011; Carvalho et al., 2011; Wagner et al., 2010). It has been suggested that the Cys497 and selenocysteine 498 residues in reduced TrxR will be the first moieties to be affected by MeHg because of the presence of selenol groups in the selenocysteine residue (Carvalho et al., 2011), but the results obtained with MeHg-inhibited TrxR indicate that the presence of selenite cannot prevent the loss of activity caused by MeHg.

There is little doubt that GSH suppresses the S-mercuration of proteins inside cells by forming a MeHg–GSH complex (MeHg–SG). There is a cellular response to MeHg through the activation of transcription factor NF-E2-related factor 2 (Nrf2) coupled to the S-mercuration of its negative regulator, Kelch-like ECH-associated protein 1 (Keap1) (Kumagai et al., 2013) to promote the formation of MeHg–SG and its excretion into extracellular space. A very interesting aspect of “thiol biology” has recently been described. We have reported that reactive sulfur species (RSS) such as hydrogen sulfide anions (HS−), persulfides, and polysulfides, have critical regulatory functions by performing redox signaling and by scavenging electrophiles (Ida et al., 2014; Nishida et al., 2012). In fact, HS− (Yoshida et al., 2011), glutathione persulfide (GS−SH) and glutathione trisulfide (GS−S−SG) (E. Yoshida, unpublished observations) as models for endogenous RSS can react with MeHg to form bismethylmercury sulfide ((MeHg)2S), which has little capability to S-mercureate proteins and is much less cytotoxic than MeHg.

Metallothioneins (MTs) are another group of proteins that are known to play roles protecting cells. Because MTs have very high affinities for MeHg by virtue of the large numbers of thiol groups (–SH) content (Oliveira et al., 1998), the formation of MeHg–MT complexes may keep MeHg in a relatively nontoxic form, thereby protecting cells from the cytotoxic effects of MeHg (Aschner et al., 1997). Decreased concentrations of GSH and/or MT could cause cells to be more susceptible to the effects of MeHg (Hirooka et al., 2010), and the disturbances in cellular signal transduction pathways could cause adverse events including cell death.

Changes in intracellular calcium ion concentrations and S-mercuration

The Ca2+ play an important role in signal transduction pathways, which affect every aspect of the life and death of a cell (Clapham, 2007). The Ca2+ concentration is normally about 10,000 times higher outside cells than in cytoplasm. Ca2+ concentrations are also much higher in some intracellular organelles, such as the endoplasmic reticulum (ER), than in cytoplasm. It has been found in a number of studies that MeHg causes the intracellular Ca2+ concentration to increase, and this effect is possibly caused by an influx of Ca2+ from outside the cells and by the release of Ca2+ from intracellular stores, such as the ER and the mitochondria (Dreiem and Seegal, 2007; Hare et al., 1993; Limke and Atchison, 2002; Limke et al., 2003; Minnema et al., 1989; Sarafian, 1993; Tan et al., 1993). The disruption of Ca2+ handling could ultimately lead to cell death. The increase in the intracellular Ca2+ concentration is believed to be caused by increased permeability of the plasma membrane and intracellular membranes to Ca2+. In addition to their lipid components, biological membranes contain proteins, so the S-mercuration of proteins involved in the transport of cations, includ-
ing Ca^{2+}, could affect this transport across the membrane, increasing the permeability of the membrane and disrupting cellular functions (Jacob and Jandl, 1962). Ca^{2+}-ATPase pumps in plasma membranes and in ER membranes maintain a low Ca^{2+} concentration by transporting Ca^{2+} away from the cytosol. Chiu et al. (1983) and Freitas et al. (1996) found that MeHg inhibits Ca^{2+} pumps such as Ca^{2+}-ATPase presumably through S-mercuration, and that this results in the Ca^{2+} fluxes across the membranes being altered. Na^+/K^+-ATPase indirectly regulates intracellular functions (Na^+ and K^+ gradients). Ca^{2+}-ATPase presumably through S-mercuration of the protein. It has also been shown that exposing rats to MeHg results in the Na^+/K^+-ATPase activity in the cerebral cortex being inhibited (Chiu et al., 2001). Therefore, it can be concluded that MeHg has the potential to inhibit signal transduction activity by S-mercuration transport proteins that are involved in the Ca^{2+} messenger functions. Interestingly, the results of a study performed by Berstein et al. (1988) suggested that the redox states of the thiols groups in muscarinic receptor molecules are important in determining their affinities for ligands. Since then, it has been found that MeHg inhibits muscarinic receptors (Coccini et al., 2000) and that MeHg can interact with muscarinic receptors to cause Ca^{2+} to be released from the ER (Limke et al., 2004). Sirois and Atchison (2000) and Peng et al. (2002) showed that MeHg affects Ca^{2+} channels, but the mechanism responsible for this effect is still unknown. It is interesting to note that Ca^{2+} channel blockers were found to prevent the body weight from decreasing and the appearance of symptoms of neurological disorders in rats treated with MeHg (Sakamoto et al., 1996). It is well recognized that disrupting calcium homeostasis in the ER causes ER stress (Mekahli et al., 2011). Consistent with this, MeHg was found to induce the expression of glucose-regulated protein 78, a maker of ER stress, in the brain cortex of rats (Zhang et al., 2013).

After the initial increase in the intracellular Ca^{2+} concentration caused by cells being exposed to MeHg, modest increases in intracellular Zn^{2+} have been found (Atchison and Hare, 1994; Denny and Atchison, 1996), although the relevance of this finding to the toxicity of MeHg is unknown. The extra free Zn^{2+} are potentially derived from proteins that contain Zn^{2+} being S-merccurated by MeHg, because under normal conditions the free Zn^{2+} concentrations in cells are low and most Zn^{2+} are bound to intracellular proteins (Cuajungco and Lees, 1997). As an example, we will later describe the S-mercuration of the zinc metalloenzymie sorbitol dehydrogenase (SDH), which leads to Zn^{2+} being released from the active site in the SDH.

Taking all of the available information into account, it appears that the S-mercuration of cellular proteins by MeHg is possibly associated with increased intracellular Ca^{2+} concentrations, and this is believed to be an important factor in the onset of MeHg toxicity.

**The inhibition of glutamate uptake and S-mercuration**

It is widely known that MeHg alters glutamate homeostasis (Farina et al., 2003a, 2003b; Manfroi et al., 2004; Yin et al., 2007). Glutamate is the predominant excitatory neurotransmitter, but it is also a potent neurotoxin when it is present in excess. MeHg inhibits the glutamate transporters GLAST (EAAT-1) and GLT-1 (EAAT-2) in astrocytes, leading to a decrease in glutamate uptake and an increase in the glutamate concentration in the extracellular fluid (Aschner et al., 2000). This could sensitize neurons to excitotoxic injury.

Sulfhydryl groups in membrane proteins have been shown to play critical functional roles in the membrane behavior (Aizenman et al., 1989; Braestrup and Andersen, 1987; Kiskin et al., 1986; Laube et al., 1993; Ruppersberg et al., 1991; Sidhu et al., 1986). The mechanisms associated with the inhibition of glutamate uptake by MeHg are unknown, but Trotti et al. (1997a, 1997b, 1998) found that the redox interconversion of cysteines in glutamate transporters (including GLAST and GLT1), induced by the thiol oxidizing agent 5,5'-dithio-bis(2-nitrobenzoic) acid and the thiol reducing agent dithiothreitol, causes changes in glutamate uptake rates. The glutamine/glutamate transporter (ASCT2) has also been found to be inhibited by MeHg through S-mercuration (Oppedisano et al., 2010). Thus, the inhibition of glutamate uptake is thought to be attributable, at least in part, to the S-mercuration of glutamate transporters. MeHg also causes increased amounts of other neurotransmitters, such as dopamine and acetylcholine, to be released (Faro et al., 2000, 2002; Minnema et al., 1989), and it has been suggested that the S-mercuration of membrane proteins may cause this effect (Faro et al., 2005; Fonfría et al., 2001).

**The inhibition of protein synthesis and S-mercuration**

It has long been known that protein synthesis is inhibited by MeHg before neurological symptoms can be found (Cavanagh and Chen, 1971; Yoshino et al., 1966). This is believed to be primarily caused by MeHg inhibiting the activities of aminoacyl-tRNA synthetases (aaRSs) (Cheung and Verity, 1985), which are enzymes that charge specific tRNAs with their cognate amino acids and
play essential roles in the initial steps involved in protein synthesis. The thiol groups in aaRSs are involved in the activities of the enzymes (Murayama et al., 1975). The mechanism through which MeHg inhibits aaRS activity has yet to be determined, but the S-mercuration of aaRSs by MeHg is thought to be associated, at least in part, with the inhibition of protein synthesis.

The disruption of microtubules and S-mercuration

Microtubules are structural components that are important in the cell division process, and also play essential roles in the maintenance of cell shape and cell motility. Administering MeHg to rats resulted in disturbances in the integrity of the microtubules in the nervous system (Kinoshita et al., 1999). It has also been found that MeHg causes the abnormal migration of neurons in the brain (Choi et al., 1978). The arresting of the G2/M phase because of microtubules being disrupted may be an important factor in MeHg leading to apoptosis (Miura K., et al., 1999). MeHg has long been known to be a potent inhibitor of microtubule polymerization, and this effect is presumably caused by the S-mercuration of the microtubules by MeHg (Abe et al., 1975; Sager et al., 1983; Vogel et al., 1985, 1989). Vogel et al. (1989) found that there is a class of binding sites that have high affinities for MeHg on tubulin and that MeHg binds to tubulin stoichiometrically within microtubules. Tubulin has been found to contain thiols with low pKa values (Britto et al., 2002).

ATTEMPTS TO IDENTIFY CASES OF THE S-MERCURATION OF PROTEINS

The biological factors that are altered by MeHg have been explored with the aim of shedding light on the molecular mechanisms that underlie MeHg toxicity (Hwang et al., 2011a, 2013a; Hwang and Naganuma, 2006; Kim et al., 2012, 2013; Lee et al., 2012; Takahashi et al., 2013; Toyama et al., 2011). Furthermore, the research group led by Dr. Naganuma has found a large number of proteins that are resistant or hypersensitive to MeHg (Hwang et al., 2007, 2009, 2010a, 2010b, 2010c, 2011b, 2012a, 2012b, 2013b). For example, they found that L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT), which is an essential enzyme for catalyzing the synthesis of glucosamine-6-phosphate from glutamine and fructose-6-phosphate, is a factor that confers MeHg-resistance and that GFAT is the intracellular target molecule for MeHg toxicity (Miura N. et al., 1999; Naganuma et al., 2000). The effects of MeHg on the activities of GFAT and other SH-containing enzymes were compared in that work, and the GFAT activity was found to be almost completely inhibited by MeHg when almost no effects on the other SH-containing enzymes were found. This suggests that MeHg has a high affinity for GFAT and specifically inhibits its activity.

As mentioned above, the true cause of MeHg toxicity is likely to be the S-mercuration of proteins. However, few studies have been performed using a “chemical biology approach” even though such studies will be required to absolutely identify the cause of MeHg toxicity. One reason for the lack of such studies is that methods for identifying the S-mercuration of proteins by MeHg have not been available. A method in which the cysteine-alkylating agent N-ethylmaleimide (NEM) is used indirectly or the radiolabeled compound is used directly has only become available until recent years. We used a new method for detecting the S-mercuration of proteins (involving column chromatography, a biotin-PEAC-maleimide (BPM)-labeling assay, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)) to screen for proteins that are targeted by MeHg, focusing on the conformational changes found in proteins when they become S-mercurated and then aggregated. There is not enough evidence to identify the toxicological effects of MeHg in the liver because the main concern regarding MeHg exposure is neurotoxicity, but MeHg has been found to accumulate strongly in the liver as well as in the brain (Yasutake et al., 1997). Therefore, we attempted to detect new target proteins for MeHg in the liver. We will introduce each approach and the proteins that were screened below.

Arginase I

Arginase I is expressed almost exclusively in the liver, where it is an essential enzyme in the urea cycle, which detoxifies ammonia. Arginase I has a binuclear Mn cluster that provides full catalytic activity and stability to the enzyme. We hypothesized that MeHg could interact with arginase I through covalent modification, resulting in alterations in not only its catalytic activity but also in the Mn concentration in the liver when in vivo exposure to MeHg occurs. Both arginase I activities and Mn concentrations in the liver fell to approximately 50% of the control values (Kanda et al., 2008). With purified arginase I, we found that interaction of MeHg with arginase I caused the aggregation of arginase I as evaluated by centrifugation and subsequent precipitation, and the catalytic activity of the arginase I to decrease. This same phenomenon occurred when NEM was used instead of MeHg, suggesting that the covalent modification of thiolate ions in rat liver arginase I led to the aggregation of the protein. Mn
ions were released from purified arginase I when its activity was inhibited by exposure to MeHg in vitro. To confirm the possibility that MeHg is able to react with reactive thiols in arginase I, we examined the elution behavior of arginase I using Affi-Gel 501 resin coupled to organomercurial (Kanda et al., 2008) (Fig. 3). When purified arginase I was poured onto a column packed with Affi-Gel 501, this protein was tightly bound to the resin. No protein was eluted by 0.5 M NaCl followed by 10 mM histidine and then 0.05% Tween 20. This suggests that the interactions of arginase I with organomercurial were not non-selective binding, hydrophobic interactions, or covalent binding via imidazol groups. However, arginase I was eluted efficiently by 10 mM 2-mercaptoethanol (2-ME), supporting the conclusion that the arginase I was covalently attached to the organomercurial through the reactive thiols in the arginase I. Further research will be required to elucidate the relationship between decreasing arginase I activities, decreasing Mn concentrations in the liver, and MeHg-dependent hepatotoxicity.

Sorbitol dehydrogenase

In the experiments that we investigated the effect of MeHg on hepatic arginase I described above, we coincidentally detected other proteins in the same hepatic preparations that also readily underwent covalent modification by MeHg and subsequently became aggregated (Kanda et al., 2012). The rat hepatic preparation was incubated with different concentrations of MeHg (1-200 μM) at 37°C for 1 hr, and then each mixture was centrifuged at 12,000 × g for 10 min. The precipitate was dissolved in 1 mM GSH (Fig. 4), and the supernatant was electrophoresed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Four main protein bands, with molecular weights of 42, 39, 30.5, and 30 kDa, were detected (Fig. 4). Similar results were found when the thiol-modifying agent NEM was used instead of MeHg, which suggests that the MeHg-mediated aggregation of the proteins was caused by the covalent modification of the protein through its reactive thiol groups. The immunoblot analysis revealed that the protein with a molecular weight of 39 kDa was arginase I. To identify the major protein with a molecular weight of 42 kDa, we separated the protein using a two-dimensional (2D) SDS-PAGE and then performed peptide mass fingerprinting using MALDI-TOF/MS. The protein with a molecular weight of 42 kDa was identified as being SDH, which is a zinc metalloenzyme that catalyzes the conversion of sorbitol into fructose in the presence of NAD+, which is a cofactor.

Using recombinant rat SDH, we found that MeHg causes (1) the protein to aggregate, (2) zinc ion to be released from the active site, and (3) the catalytic activity to be inhibited. These MeHg-dependent phenomena were blocked when GSH was added, suggesting that the S-mercuration of SDH contributed to the effects that were observed. Consistent with this notion, SDH was found to be easily modified by MeHg in tests using the BPM-labeling assay (Toyama et al., 2013) (Fig. 5). In this assay,
Protein S-mercuration and toxic effects by methylmercury

Fig. 4. Screening to identify proteins that underwent aggregation through S-mercuration by MeHg and the target molecules obtained from rat liver preparations. The hepatic preparation was incubated with MeHg for 1 hr at 37°C, then the mixture was centrifuged at 12,000 × g for 10 min, and the precipitate was dissolved in 1 mM GSH. The precipitate was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were detected using the silver staining technique. The lane numbers 1, 2, 3, 4, and 5 correspond to MeHg concentrations of 0, 1, 10, 100, and 200 μM, respectively. Reprinted from Kanda et al., 2012.

Fig. 5. BPM-labeling assay used to analyze rat SDH treated with MeHg. The assay was used to detect BPM-modified thiols in proteins that had been exposed to MeHg. Recombinant rat SDH (0.3 mg/mL) was incubated with MeHg (10 or 25 μM) at 37°C for 1 hr. Reprinted from Kanda et al., 2012.
protein S-mercuration by MeHg can be estimated by a decrease in protein modification by BPM, which is a thiol reactive probe. BPM is a biotinylated-maleimide that is often used to visualize reactive protein thiols using horseradish peroxidase-conjugated streptavidin (avidin-HRP), taking advantage of a better avidin-binding and resistance to extreme changes in temperature and pH. BPM is commercially available and does not require a specific antibody against MeHg. In this system, a decrease in the amount of BPM-binding that occurs reflects a decrease in the number of free thiol groups available. It has been reported that rat SDH has 10 cysteine residues in a subunit, but the MALDI-TOF/MS results suggested that the rat SDH was S-mercured by MeHg in four cysteine residues, Cys44, Cys119, Cys129, and Cys164 (Fig. 6).

Serine mutations in Cys44 and Cys129 dramatically blocked the MeHg-mediated aggregation of rat SDH. This suggests that alterations in the three-dimensional structure of this protein that are caused by S-mercuration may have been responsible for facilitating the substantial aggregation that was observed. Since Cys44 functions as a ligand for the coordination of the active site zinc ion, it seems likely that the S-mercuration of Cys44 will be a major cause of the release of zinc ion. In our preliminary study, we found that exposing primary mouse hepatocytes to MeHg resulted in a negligible decrease in cellular SDH activity. Since pretreatment with NAD, which binds closely to Cys44, caused the concentration-dependent inhibition of SDH aggregation by MeHg, the lack of a decrease in cellular SDH activity in the hepatocytes may have been caused by the protective effects of cellular factors not only GSH but also NAD on the covalent modification of cellular SDH by MeHg.

Glutathione S-transferase

We also tried to isolate the proteins with molecular weights of 30 and 30.5 kDa using 2D SDS-PAGE and to identify the proteins using MALDI-TOF/MS. The 30 and 30.5 kDa proteins were glutathione S-transferase (GST) Yb (GSTμ) and GSTYa (GSTα), respectively (H. Kanda, unpublished observations). GST is a phase II enzyme that detoxifies a broad range of electrophiles by conjugating them with the reduced form of GSH. The pKa of GSH is high (9.2) and little GSH is ionized at physiological pH values, but GST decreases the pKa of the thiol in GSH in the enzyme-GSH complex. It has been suggested that the cysteine residue(s) in GST are important (Cheng et al., 2001; Katusz et al., 1992), so further research is needed.

CONCLUSIONS

To truly understand MeHg toxic effects, the fundamental reason for the effects needs to be found. We found that the S-mercuration of cellular proteins, such as Mn-SOD (oxidative stress), membrane proteins such as transporters (impaired signaling), and tubulin (disruption of microtubules), is probably involved in the main mechanisms that have been suggested to underlie MeHg toxicity. Methods for identifying S-mercured proteins are increasingly becoming available, and these methods may allow the mechanisms through which S-mercuration is involved in MeHg toxicity to be understood in the future and the key cellular proteins involved to be identified.
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