Review

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

Hironori Kanda¹, Yasuhiro Shinkai² and Yoshito Kumagai²

¹Doctoral Program in Biomedical Sciences, Graduate School of Comprehensive Human Sciences,
University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

²Environmental Biology Laboratory, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

(Received July 11, 2014; Accepted July 16, 2014)

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 (Zn^{2+}). Between 10% and 20% of the thiol groups in proteins are dissociated, as thiolate anions, under physiological conditions because their *pK_a* 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} – 10^{16} ; 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).

Correspondence: Yoshito Kumagai (E-mail: yk-em-tu@md.tsukuba.ac.jp)

Table 1. Association constants for MeHg and various ligands

Ligand	Association constant (logarithm)
SH (Cysteine)	15.7
NH ₂ (Histidine)	8.8
NH ₃	8.4
Imidazole	7.3

Partially reprinted from Simpson, 1961.

Fig. 1. Unintentional toxicity caused by MeHg mediated S-mercuration. The unintentional S-mercuration of cellular proteins by MeHg at high concentrations leads to cell death, whereas the S-mercuration of redox sensor proteins could activate electrophilic signal transduction pathways for cell survival.

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.

MAJOR MECHANISMS UNDERLYING METHYLMERCURY TOXICITY

To determine the major mechanisms that have been suggested to underlie MeHg toxicity, we searched the lit-

erature 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-mercured

Mechanism and features	Factors	Articles	Potential S-mercured proteins	Reference
1. Oxidative stress (increased reactive oxygen species (ROS); decreased endogenous antioxidant defense)	<ul style="list-style-type: none"> ● 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+} 	<p>Fonnum and Lock, 2004; Patel and Reynolds, 2013; Shanker and Aschner, 2001</p>	Mn-SOD	Kumagai <i>et al.</i> , 1997; Shinyashiki <i>et al.</i> , 1996
2. Changes in intracellular concentrations of Ca^{2+}	<ul style="list-style-type: none"> ● Increase in plasma membrane permeability (Inhibition of Ca^{2+} channels and $\text{Na}^{+}/\text{K}^{+}$-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 	<p>Atchison and Hare, 1994; Denny and Atchison, 1996; Fonnum and Lock, 2004</p>	Ca^{2+} -ATPase $\text{Na}^{+}/\text{K}^{+}$ -ATPase Muscarinic receptor (?) ^a Ca^{2+} channel (?) ^a	<p>Freitas <i>et al.</i>, 1996 Magour, 1986 Coccini <i>et al.</i>, 2000; Limke <i>et al.</i>, 2004 Peng <i>et al.</i>, 2002; Sirois and Atchison, 2000</p>
3. Inhibition of glutamate uptake	<ul style="list-style-type: none"> ● Inhibition of glutamate transporters (EAAT-1/GLAST and EAAT-2/GLT-1) 	<p>Aschner, 2000; Aschner <i>et al.</i>, 2000; Fonnum and Lock, 2004</p>	EAAT-1 (GLAST) (?) ^a EAAT-2 (GLT-1) (?) ^a ASCT2 (?) ^a	<p>Aschner <i>et al.</i>, 2000 Aschner <i>et al.</i>, 2000 Oppedisano <i>et al.</i>, 2010</p>
4. Inhibition of protein synthesis	<ul style="list-style-type: none"> ● Direct interaction with some parts of the protein synthetic machinery (including inhibition of the aminoacyl-tRNA synthetase enzymes) 	<p>Atchison and Hare, 1994; Clarkson, 1987; Miura and Imura, 1987</p>	Aminoacyl-tRNA synthetase (?) ^a	Cheung and Verity, 1985
5. Disruption of microtubules	<ul style="list-style-type: none"> ● Reaction with SH groups in tubulin 	<p>Clarkson, 1987; Miura and Imura, 1987</p>	Tubulin	Vogel <i>et al.</i> , 1985, 1989

^a Tentative

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 (Araie 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-associat-

ed 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-mercure 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 Ca^{2+} concentrations by controlling the Na^+ gradient. Magour (1986) found that MeHg inhibits brain synaptosomal Na^+/K^+ -ATPase, presumably by *S*-mercurating 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 (Chuu *et al.*, 2001). Therefore, it can be concluded that MeHg has the potential to inhibit signal transduction activity by *S*-mercurating 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 thiol 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 marker 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*-mercurated 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 metalloenzyme 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; Fonfria *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 integrities 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-PEAC₅-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-mercured 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 μ M)

at 37°C for 1 hr, and then each mixture was centrifuged at $12,000 \times g$ 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-mercuriation 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,

Fig. 3. Schematic of the elution of purified arginase I from an arylmercury column. The purified arginase I protein was applied to the organomercurial column (Affi-Gel 501; 2.0×1.0 cm, i.d.) and the proteins that bound to the column were eluted using 0.5 M NaCl, then 10 mM histidine, 0.05% Tween 20, and finally 10 mM 2-mercaptoethanol.

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 $12,000 \times g$ 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.

Fig. 6. Sites of MeHg modification in rat SDH. The 3D structure actually shows sheep liver SDH (from the PROTEIN DATA BANK). The image and the color modifications to indicate the cysteine residues were produced using RasMol. The residues in yellow are the sites where MeHg modifications occurred and the residues in blue are other cysteines. The cysteine residues of rat liver SDH are numbered based on amino-acid sequence homology. The atom in red is zinc.

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 (GST μ) and GSTYa (GST α), 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 pK_a of GSH is high (9.2) and little GSH is ionized at physiological pH values, but GST decreases the pK_a 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.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid (#25220103) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Abe, T., Haga, T. and Kurokawa, M. (1975): Blockage of axoplasmic transport and depolymerisation of reassembled microtubules by methyl mercury. *Brain Res.*, **86**, 504-508.
- Aizenman, E., Lipton, S.A. and Loring, R.H. (1989): Selective modulation of NMDA responses by reduction and oxidation. *Neuron*, **2**, 1257-1263.
- Ali, S.F., LeBel, C.P. and Bondy, S.C. (1992): Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. *Neurotoxicology*, **13**, 637-648.
- Araie, H. and Shiraiwa, Y. (2009): Selenium utilization strategy by microalgae. *Molecules*, **14**, 4880-4891.
- Aschner, M. (2000): Astrocytic swelling, phospholipase A2, glutathione and glutamate: interactions in methylmercury-induced neurotoxicity. *Cell. Mol. Biol.*, **46**, 843-854.
- Aschner, M., Cherian, M.G., Klaassen, C.D., Palmiter, R.D., Erickson, J.C. and Bush, A.I. (1997): Metallothioneins in brain—the role in physiology and pathology. *Toxicol. Appl. Pharmacol.*, **142**, 229-242.
- Aschner, M., Yao, C.P., Allen, J.W. and Tan, K.H. (2000): Methylmercury alters glutamate transport in astrocytes. *Neurochem. Int.*, **37**, 199-206.
- Atchison, W.D. and Hare, M.F. (1994): Mechanisms of methylmercury-induced neurotoxicity. *FASEB J.*, **8**, 622-629.
- Berstein, G., Haga, K., Haga, T. and Ichiyama, A. (1988): Agonist and antagonist binding of muscarinic acetylcholine receptors purified from porcine brain: interconversion of high- and low-affinity sites by sulfhydryl reagents. *J. Neurochem.*, **50**, 1687-1694.
- Braestrup, C. and Andersen, P.H. (1987): Effects of heavy metal cations and other sulfhydryl reagents on brain dopamine D1 receptors: evidence for involvement of a thiol group in the conformation of the active site. *J. Neurochem.*, **48**, 1667-1672.
- Branco, V., Canário, J., Holmgren, A. and Carvalho, C. (2011): Inhibition of the thioredoxin system in the brain and liver of zebra-seabreams exposed to waterborne methylmercury. *Toxicol. Appl. Pharmacol.*, **251**, 95-103.
- Britto, P.J., Knipling, L. and Wolff, J. (2002): The local electrostatic environment determines cysteine reactivity of tubulin. *J. Biol. Chem.*, **277**, 29018-29027.
- Carvalho, M.C., Franco, J.L., Ghizoni, H., Kobus, K., Nazari, E.M., Rocha, J.B., Nogueira, C.W., Dafre, A.L., Müller, Y.M. and Farina, M. (2007): Effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) on methylmercury-induced locomotor deficits and cerebellar toxicity in mice. *Toxicology*, **239**, 195-203.
- Carvalho, C.M., Lu, J., Zhang, X., Arnér, E.S. and Holmgren, A. (2011): Effects of selenite and chelating agents on mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning. *FASEB J.*, **25**, 370-381.
- Cavanagh, J.B. and Chen, F.C. (1971): Amino acid incorporation in protein during the “silent phase” before organo-mercury and *p*-bromophenylacetylurea neuropathy in the rat. *Acta Neuropathol.*, **19**, 216-224.
- Chang, L.W. (1990): The neurotoxicology and pathology of organomercury, organolead, and organotin. *J. Toxicol. Sci.*, **15**, 125-151.
- Cheng, H., Tchaikovskaya, T., Tu, Y.S., Chapman, J., Qian, B., Ching, W.M., Tien, M., Rowe, J.D., Patskovsky, Y.V., Listowsky, I. and Tu, C.P. (2001): Rat glutathione S-transferase M4-4: an isoenzyme with unique structural features including a redox-reactive cysteine-115 residue that forms mixed disulphides with glutathione. *Biochem. J.*, **356**, 403-414.
- Cheung, M.K. and Verity, M.A. (1985): Experimental methyl mercury neurotoxicity: locus of mercurial inhibition of brain protein synthesis *in vivo* and *in vitro*. *J. Neurochem.*, **44**, 1799-1808.
- Chiu, V.C., Mouring, D. and Haynes, D.H. (1983): Action of mercurials on the active and passive transport properties of sarcoplasmic reticulum. *J. Bioenerg. Biomembr.*, **15**, 13-25.
- Choi, B.H., Lapham, L.W., Amin-Zaki, L. and Saleem, T. (1978): Abnormal neuronal migration, deranged cerebral cortical organization, and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning in utero. *J. Neuropathol. Exp. Neurol.*, **37**, 719-733.
- Chuu, J.J., Liu, S.H. and Lin-Shiau, S.Y. (2001): Effects of methyl mercury, mercuric sulfide and cinnabar on active avoidance responses, Na⁺/K⁺-ATPase activities and tissue mercury contents in rats. *Proc. Natl. Sci. Counc. Repub. China B*, **25**, 128-136.
- Clapham, D.E. (2007): Calcium signaling. *Cell*, **131**, 1047-1058.
- Clarkson, T.W. (1987): Metal toxicity in the central nervous system. *Environ. Health Perspect.*, **75**, 59-64.
- Coccini, T., Randine, G., Candura, S.M., Nappi, R.E., Prockop, L.D. and Manzo, L. (2000): Low-level exposure to methylmercury modifies muscarinic cholinergic receptor binding characteristics in rat brain and lymphocytes: physiologic implications and new opportunities in biologic monitoring. *Environ. Health Perspect.*, **108**, 29-33.
- Committee on the Toxicological Effects of Methylmercury (2000): Toxicological effects of methylmercury, pp.55, National Academy Press. Washington, DC.
- Cuajungco, M.P. and Lees, G.J. (1997): Zinc metabolism in the brain: relevance to human neurodegenerative disorders. *Neurobiol. Dis.*, **4**, 137-169.
- Denny, M.F. and Atchison, W.D. (1996): Mercurial-induced alterations in neuronal divalent cation homeostasis. *Neurotoxicology*, **17**, 47-61.
- Dreiem, A. and Seegal, R.F. (2007): Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent. *Neurotoxicology*, **28**, 720-726.
- Farina, M., Aschner, M. and Rocha, J.B. (2011): Oxidative stress in MeHg-induced neurotoxicity. *Toxicol. Appl. Pharmacol.*, **256**, 405-417.
- Farina, M., Campos, F., Vendrell, I., Berenguer, J., Barzi, M., Pons, S. and Suñol, C. (2009): Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. *Toxicol. Sci.*, **112**, 416-426.
- Farina, M., Dahm, K.C., Schwalm, F.D., Brusque, A.M., Frizzo, M.E., Zeni, G., Souza, D.O. and Rocha, J.B. (2003a): Methylmercury increases glutamate release from brain synaptosomes and

- glutamate uptake by cortical slices from suckling rat pups: modulatory effect of ebselen. *Toxicol. Sci.*, **73**, 135-140.
- Farina, M., Frizzo, M.E., Soares, F.A., Schwalm, F.D., Dietrich, M.O., Zeni, G., Rocha, J.B. and Souza, D.O. (2003b): Ebselen protects against methylmercury-induced inhibition of glutamate uptake by cortical slices from adult mice. *Toxicol. Lett.*, **144**, 351-357.
- Faro, L.R., do Nascimento, J.L., Alfonso, M. and Durán, R. (2002): Mechanism of action of methylmercury on *in vivo* striatal dopamine release. Possible involvement of dopamine transporter. *Neurochem. Int.*, **40**, 455-465.
- Faro, L.R., do Nascimento, J.L., Campos, F., Vidal, L., Alfonso, M. and Durán, R. (2005): Protective effects of glutathione and cysteine on the methylmercury-induced striatal dopamine release *in vivo*. *Life Sci.*, **77**, 444-451.
- Faro, L.R., do Nascimento, J.L., San José, J.M., Alfonso, M. and Durán, R. (2000): Intra-striatal administration of methylmercury increases *in vivo* dopamine release. *Neurochem. Res.*, **25**, 225-229.
- Fonfría, E., Rodríguez-Farré, E. and Suñol, C. (2001): Mercury interaction with the GABA(A) receptor modulates the benzodiazepine binding site in primary cultures of mouse cerebellar granule cells. *Neuropharmacology*, **41**, 819-833.
- Fonnum, F. and Lock, E.A. (2004): The contributions of excitotoxicity, glutathione depletion and DNA repair in chemically induced injury to neurones: exemplified with toxic effects on cerebellar granule cells. *J. Neurochem.*, **88**, 513-531.
- Franco, J.L., Posser, T., Dunkley, P.R., Dickson, P.W., Mattos, J.J., Martins, R., Bainy, A.C., Marques, M.R., Dafre, A.L. and Farina, M. (2009): Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. *Free Radic. Biol. Med.*, **47**, 449-457.
- Freitas, A.J., Rocha, J.B., Wolosker, H. and Souza, D.O. (1996): Effects of Hg^{2+} and CH_3Hg^+ on Ca^{2+} fluxes in rat brain microsomes. *Brain Res.*, **738**, 257-264.
- Garg, T.K. and Chang, J.Y. (2006): Methylmercury causes oxidative stress and cytotoxicity in microglia: attenuation by 15-deoxy-delta 12, 14-prostaglandin J2. *J. Neuroimmunol.*, **171**, 17-28.
- Glaser, V., Nazari, E.M., Müller, Y.M., Feksa, L., Wannmacher, C.M., Rocha, J.B., de Bem, A.F., Farina, M. and Latini, A. (2010): Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex. *Int. J. Dev. Neurosci.*, **28**, 631-637.
- Halliwel, B. (1992): Reactive oxygen species and the central nervous system. *J. Neurochem.*, **59**, 1609-1623.
- Hare, M.F., McGinnis, K.M. and Atchison, W.D. (1993): Methylmercury increases intracellular concentrations of Ca^{++} and heavy metals in NG108-15 cells. *J. Pharmacol. Exp. Ther.*, **266**, 1626-1635.
- Hirooka, T., Fujiwara, Y., Shinkai, Y., Yamamoto, C., Yasutake, A., Satoh, M., Eto, K. and Kaji, T. (2010): Resistance of human brain microvascular endothelial cells in culture to methylmercury: cell-density-dependent defense mechanisms. *J. Toxicol. Sci.*, **35**, 287-294.
- Hirota, Y., Yamaguchi, S., Shimojoh, N. and Sano, K.I. (1980): Inhibitory effect of methylmercury on the activity of glutathione peroxidase. *Toxicol. Appl. Pharmacol.*, **53**, 174-176.
- Hwang, G.W., Hayashi, T., Kita, K., Takahashi, T., Kuge, S. and Naganuma, A. (2007): siRNA-mediated inhibition of phosphatidylinositol glycan Class B (PIGB) confers resistance to methylmercury in HEK293 cells. *J. Toxicol. Sci.*, **32**, 581-583.
- Hwang, G.W., Kimura, Y., Takahashi, T., Lee, J.Y. and Naganuma, A. (2012a): Identification of deubiquitinating enzymes involved in methylmercury toxicity in *Saccharomyces cerevisiae*. *J. Toxicol. Sci.*, **37**, 1287-1290.
- Hwang, G.W., Lee, J.Y., Kim, M.S., Sato, M., Takahashi, T. and Naganuma, A. (2013a): Changes in the levels of low molecular weight metabolites in the mouse cerebellum following treatment with methylmercury. *J. Toxicol. Sci.*, **38**, 703-706.
- Hwang, G.W., Lee, J.Y., Ryoike, K., Matsuyama, F., Kim, J.M., Takahashi, T. and Naganuma, A. (2011a): Gene expression profiling using DNA microarray analysis of the cerebellum of mice treated with methylmercury. *J. Toxicol. Sci.*, **36**, 389-391.
- Hwang, G.W., Mastuyama, F., Takahashi, T., Lee, J.Y. and Naganuma, A. (2013b): Deletion of the ubiquitin-conjugating enzyme Ubc2 confers resistance to methylmercury in budding yeast by promoting Whi2 degradation. *J. Toxicol. Sci.*, **38**, 301-303.
- Hwang, G.W. and Naganuma, A. (2006): DNA microarray analysis of transcriptional responses of human neuroblastoma IMR-32 cells to methylmercury. *J. Toxicol. Sci.*, **31**, 537-538.
- Hwang, G.W., Ogiwara, Y., Takahashi, T. and Naganuma, A. (2012b): Ubiquitin-conjugating enzyme Cdc34 mediates methylmercury resistance in *Saccharomyces cerevisiae* by increasing Whi2 degradation. *J. Toxicol. Sci.*, **37**, 1283-1286.
- Hwang, G.W., Oh, S.E., Takahashi, T., Lee, J.Y. and Naganuma, A. (2010a): siRNA-mediated knockdown of the melanocortin 2 receptor accessory protein 2 (MRAP2) gene confers resistance to methylmercury on HEK293 cells. *J. Toxicol. Sci.*, **35**, 947-950.
- Hwang, G.W., Ryoike, K., Lee, J.Y., Takahashi, T. and Naganuma, A. (2011b): siRNA-mediated silencing of the gene for heat shock transcription factor 1 causes hypersensitivity to methylmercury in HEK293 cells. *J. Toxicol. Sci.*, **36**, 851-853.
- Hwang, G.W., Ryoike, K., Takahashi, T. and Naganuma, A. (2010b): Silencing of the gene for homeobox protein HOXB13 by siRNA confers resistance to methylmercury on HEK293 cells. *J. Toxicol. Sci.*, **35**, 941-944.
- Hwang, G.W., Tobita, M., Takahashi, T., Kuge, S., Kita, K. and Naganuma, A. (2010c): siRNA-mediated AMPK α 1 subunit gene PRKAA1 silencing enhances methylmercury toxicity in HEK293 cells. *J. Toxicol. Sci.*, **35**, 601-604.
- Hwang, G.W., Wada, N., Kuge, S. and Naganuma, A. (2009): Overexpression of the novel F-box protein Ymr258c confers resistance to methylmercury in *Saccharomyces cerevisiae*. *J. Toxicol. Sci.*, **34**, 413-416.
- Ida, T., Sawa, T., Ihara, H., Tsuchiya, Y., Watanabe, Y., Kumagai, Y., Suematsu, M., Motohashi, H., Fujii, S., Matsunaga, T., Yamamoto, M., Ono, K., Devarie-Baez, N.O., Xian, M., Fukuto, J.M. and Akaike, T. (2014): Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proc. Natl. Acad. Sci. USA*, **111**, 7606-7611.
- Jacob, H.S. and Jandl, J.H. (1962): Effects of sulfhydryl inhibition on red blood cells. I. Mechanism of hemolysis. *J. Clin. Invest.*, **41**, 779-792.
- Jones, D.P. (2008): Radical-free biology of oxidative stress. *Am. J. Physiol. Cell Physiol.*, **295**, C849-868.
- Kanda, H., Sumi, D., Endo, A., Toyama, T., Chen, C.L., Kikushima, M. and Kumagai, Y. (2008): Reduction of arginase I activity and manganese levels in the liver during exposure of rats to methylmercury: a possible mechanism. *Arch. Toxicol.*, **82**, 803-808.
- Kanda, H., Toyama, T., Shinohara-Kanda, A., Iwamatsu, A., Shinkai, Y., Kaji, T., Kikushima, M. and Kumagai, Y. (2012): S-Mercuration of rat sorbitol dehydrogenase by methylmercury causes its aggregation and the release of the zinc ion from the active site.

- Arch. Toxicol., **86**, 1693-1702.
- Katusz, R.M., Bono, B. and Colman, R.F. (1992): Affinity labeling of Cys111 of glutathione S-transferase, isoenzyme 1-1, by S-(4-bromo-2,3-dioxobutyl)glutathione. *Biochemistry*, **31**, 8984-8990.
- Kim, M.S., Takahashi, T., Lee, J.Y., Hwang, G.W. and Naganuma, A. (2012): Methylmercury induces CCL2 expression through activation of NF- κ B in human 1321N1 astrocytes. *J. Toxicol. Sci.*, **37**, 1275-1278.
- Kim, M.S., Takahashi, T., Lee, J.Y., Hwang, G.W. and Naganuma, A. (2013): Global chemokine expression in methylmercury-treated mice: methylmercury induces brain-specific expression of CCL3 and CCL4. *J. Toxicol. Sci.*, **38**, 925-929.
- Kinoshita, Y., Ohnishi, A., Kohshi, K. and Yokota, A. (1999): Apparent diffusion coefficient on rat brain and nerves intoxicated with methylmercury. *Environ. Res.*, **80**, 348-354.
- Kiskin, N.I., Krishtal, O.A., Tsyndrenko, A.Ya and Akaike, N. (1986): Are sulfhydryl groups essential for function of the glutamate-operated receptor-ionophore complex? *Neurosci. Lett.*, **66**, 305-310.
- Kromidas, L., Trombetta, L.D. and Jamall, I.S. (1990): The protective effects of glutathione against methylmercury cytotoxicity. *Toxicol. Lett.*, **51**, 67-80.
- Kumagai, Y., Homma-Takeda, S., Shinyashiki, M. and Shimojo, N. (1997): Alterations in Superoxide Dismutase Isozymes by Methylmercury. *Appl. Organomet. Chem.*, **11**, 635-643.
- Kumagai, Y., Kanda, H., Shinkai, Y. and Toyama, T. (2013): The role of the Keap1/Nrf2 pathway in the cellular response to methylmercury. *Oxid. Med. Cell. Longev.*, **2013**, 848279.
- Kumagai, Y., Shinkai, Y., Miura, T. and Cho, A.K. (2012): The chemical biology of naphthoquinones and its environmental implications. *Annu. Rev. Pharmacol. Toxicol.*, **52**, 221-247.
- Kumagai, Y. and Sumi, D. (2007): Arsenic: signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. *Annu. Rev. Pharmacol. Toxicol.*, **47**, 243-262.
- Laube, B., Kuryatov, A., Kuhse, J. and Betz, H. (1993): Glycine-glutamate interactions at the NMDA receptor: role of cysteine residues. *FEBS Lett.*, **335**, 331-334.
- LeBel, C.P., Ali, S.F. and Bondy, S.C. (1992): Deferoxamine inhibits methyl mercury-induced increases in reactive oxygen species formation in rat brain. *Toxicol. Appl. Pharmacol.*, **112**, 161-165.
- LeBel, C.P., Ali, S.F., McKee, M. and Bondy, S.C. (1990): Organometal-induced increases in oxygen reactive species: the potential of 2',7'-dichlorofluorescein diacetate as an index of neurotoxic damage. *Toxicol. Appl. Pharmacol.*, **104**, 17-24.
- Lee, J.Y., Hwang, G.W., Kim, M.S., Takahashi, T. and Naganuma, A. (2012): Methylmercury induces a brain-specific increase in chemokine CCL4 expression in mice. *J. Toxicol. Sci.*, **37**, 1279-1282.
- Lee, J.Y., Hwang, G.W. and Naganuma, A. (2009): Rip1 enhances methylmercury toxicity through production of reactive oxygen species (ROS) in budding yeast. *J. Toxicol. Sci.*, **34**, 715-717.
- Levonon, A.L., Hill, B.G., Kansanen, E., Zhang, J. and Darley-Usmar, V.M. (2014): Redox regulation of antioxidants, autophagy, and the response to stress: Implications for electrophile therapeutics. *Free Radic. Biol. Med.*, **71C**, 196-207.
- Limke, T.L. and Atchison, W.D. (2002): Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol.*, **178**, 52-61.
- Limke, T.L., Bearss, J.J. and Atchison, W.D. (2004): Acute exposure to methylmercury causes Ca^{2+} dysregulation and neuronal death in rat cerebellar granule cells through an M3 muscarinic receptor-linked pathway. *Toxicol. Sci.*, **80**, 60-68.
- Limke, T.L., Otero-Montañez, J.K. and Atchison, W.D. (2003): Evidence for interactions between intracellular calcium stores during methylmercury-induced intracellular calcium dysregulation in rat cerebellar granule neurons. *J. Pharmacol. Exp. Ther.*, **304**, 949-958.
- Lobanov, A.V., Hatfield, D.L. and Gladyshev, V.N. (2009): Eukaryotic selenoproteins and selenoproteomes. *Biochim. Biophys. Acta.*, **1790**, 1424-1428.
- Magour, S. (1986): Studies on the inhibition of brain synaptosomal Na^+/K^+ -ATPase by mercury chloride and methyl mercury chloride. *Arch. Toxicol. Suppl.*, **9**, 393-396.
- Manfroi, C.B., Schwalm, F.D., Cereser, V., Abreu, F., Oliveira, A., Bizarro, L., Rocha, J.B., Frizzo, M.E., Souza, D.O. and Farina, M. (2004): Maternal milk as methylmercury source for suckling mice: neurotoxic effects involved with the cerebellar glutamatergic system. *Toxicol. Sci.*, **81**, 172-178.
- Matsuda, Y., Higashiyama, S., Kijima, Y., Suzuki, K., Kawano, K., Akiyama, M., Kawata, S., Tarui, S., Deutsch, H.F. and Taniguchi, N. (1990): Human liver manganese superoxide dismutase. Purification and crystallization, subunit association and sulfhydryl reactivity. *Eur. J. Biochem.*, **194**, 713-720.
- Mekahli, D., Bultynck, G., Parys, J.B., De Smedt, H. and Missiaen, L. (2011): Endoplasmic-reticulum calcium depletion and disease. *Cold Spring Harb. Perspect. Biol.*, **3**, 1-30.
- Minnema, D.J., Cooper, G.P. and Greenland, R.D. (1989): Effects of methylmercury on neurotransmitter release from rat brain synaptosomes. *Toxicol. Appl. Pharmacol.*, **99**, 510-521.
- Miura, K. and Imura, N. (1987): Mechanism of methylmercury cytotoxicity. *Crit. Rev. Toxicol.*, **18**, 161-188.
- Miura, K., Koide, N., Himeno, S., Nakagawa, I. and Imura, N. (1999): The involvement of microtubular disruption in methylmercury-induced apoptosis in neuronal and nonneuronal cell lines. *Toxicol. Appl. Pharmacol.*, **160**, 279-288.
- Miura, N., Kaneko, S., Hosoya, S., Furuchi, T., Miura, K., Kuge, S. and Naganuma, A. (1999): Overexpression of L-glutamine:D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*. *FEBS Lett.*, **458**, 215-218.
- Mori, N., Yasutake, A. and Hirayama, K. (2007): Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. *Arch. Toxicol.*, **81**, 769-776.
- Murayama, A., Raffin, J.P., Remy, P. and Ebel, J.P. (1975): Yeast phenylalanyl-tRNA synthetase: properties of the sulfhydryl groups; evidence for -SH requirement in tRNA acylation. *FEBS Lett.*, **53**, 15-22.
- Naganuma, A., Miura, N., Kaneko, S., Mishina, T., Hosoya, S., Miyairi, S., Furuchi, T. and Kuge, S. (2000): GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*. *FASEB J.*, **14**, 968-972.
- Naganuma, A., Miura, K., Tanaka-Kagawa, T., Kitahara, J., Seko, Y., Toyoda, H. and Imura, N. (1998): Overexpression of manganese-superoxide dismutase prevents methylmercury toxicity in HeLa cells. *Life Sci.*, **62**, PL157-161.
- Nishida, M., Sawa, T., Kitajima, N., Ono, K., Inoue, H., Ihara, H., Motohashi, H., Yamamoto, M., Suematsu, M., Kurose, H., van der Vliet, A., Freeman, B.A., Shibata, T., Uchida, K., Kumagai, Y. and Akaike, T. (2012): Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydrylation. *Nat. Chem.*

- Biol., **8**, 714-724.
- Oliveira, R.B., Gomes-Leal, W., do-Nascimento, J.L. and Picanço-Diniz, C.W. (1998): Methylmercury intoxication and histochemical demonstration of NADPH-diaphorase activity in the striate cortex of adult cats. *Braz. J. Med. Biol. Res.*, **31**, 1157-1161.
- Oppedisano, F., Galluccio, M. and Indiveri, C. (2010): Inactivation by Hg²⁺ and methylmercury of the glutamine/amino acid transporter (ASCT2) reconstituted in liposomes: Prediction of the involvement of a CXXC motif by homology modelling. *Biochem. Pharmacol.*, **80**, 1266-1273.
- Patel, E. and Reynolds, M. (2013): Methylmercury impairs motor function in early development and induces oxidative stress in cerebellar granule cells. *Toxicol. Lett.*, **222**, 265-272.
- Peng, S., Hajela, R.K. and Atchison, W.D. (2002): Effects of methylmercury on human neuronal L-type calcium channels transiently expressed in human embryonic kidney cells (HEK-293). *J. Pharmacol. Exp. Ther.*, **302**, 424-432.
- Ruppersberg, J.P., Stocker, M., Pongs, O., Heinemann, S.H., Frank, R. and Koenen, M. (1991): Regulation of fast inactivation of cloned mammalian IK(A) channels by cysteine oxidation. *Nature*, **352**, 711-714.
- Sager, P.R., Doherty, R.A. and Olmsted, J.B. (1983): Interaction of methylmercury with microtubules in cultured cells and *in vitro*. *Exp. Cell Res.*, **146**, 127-137.
- Sakamoto, M., Ikegami, N. and Nakano, A. (1996): Protective effects of Ca²⁺ channel blockers against methyl mercury toxicity. *Pharmacol. Toxicol.*, **78**, 193-199.
- Sarafian, T.A. (1993): Methyl mercury increases intracellular Ca²⁺ and inositol phosphate levels in cultured cerebellar granule neurons. *J. Neurochem.*, **61**, 648-657.
- Shanker, G. and Aschner, M. (2001): Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. *J. Neurosci. Res.*, **66**, 998-1002.
- Shimai, S. and Satoh, H. (1985): Behavioral teratology of methylmercury. *J. Toxicol. Sci.*, **10**, 199-216.
- Shinyashiki, M., Kumagai, Y., Homma-Takeda, S., Nagafune, J., Takasawa, N., Suzuki, J., Matsuzaki, I., Satoh, S., Sagai, M. and Shimojo, N. (1996): Selective inhibition of the mouse brain Mn-SOD by methylmercury. *Environ. Toxicol. Pharmacol.*, **2**, 359-366.
- Sidhu, A., Kassis, S., Kebabian, J. and Fishman, P.H. (1986): Sulfhydryl group(s) in the ligand binding site of the D-1 dopamine receptor: specific protection by agonist and antagonist. *Biochemistry*, **25**, 6695-6701.
- Simpson, R.B. (1961): Association constants of methylmercury with sulfhydryl and other bases. *J. Am. Chem. Soc.*, **83**, 4711-4717.
- Sirois, J.E. and Atchison, W.D. (2000): Methylmercury affects multiple subtypes of calcium channels in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol.*, **167**, 1-11.
- Stringari, J., Nunes, A.K., Franco, J.L., Bohrer, D., Garcia, S.C., Dafre, A.L., Milatovic, D., Souza, D.O., Rocha, J.B., Aschner, M. and Farina, M. (2008): Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain. *Toxicol. Appl. Pharmacol.*, **227**, 147-154.
- Takahashi, T., Kim, M.S., Saito, T., Lee, J.Y., Hwang, G.W. and Naganuma, A. (2013): Brain-specific induction of secretogloblin 3A1 expression in mice treated with methylmercury. *J. Toxicol. Sci.*, **38**, 963-965.
- Tan, X.X., Tang, C., Castoldi, A.F., Manzo, L. and Costa, L.G. (1993): Effects of inorganic and organic mercury on intracellular calcium levels in rat T lymphocytes. *J. Toxicol. Environ. Health*, **38**, 159-170.
- Toyama, T., Shinkai, Y., Kaji, T. and Kumagai, Y. (2013): Convenient method to assess chemical modification of protein thiols by electrophilic metals. *J. Toxicol. Sci.*, **38**, 477-484.
- Toyama, T., Yoshida, E., Shinkai, Y. and Kumagai, Y. (2011): DNA microarray analysis of human neuroblastoma SH-SY5Y cells exposed to methylmercury. *J. Toxicol. Sci.*, **36**, 843-845.
- Trotti, D., Danbolt, N.C. and Volterra, A. (1998): Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? *Trends Pharmacol. Sci.*, **19**, 328-334.
- Trotti, D., Nussberger, S., Volterra, A. and Hediger, M.A. (1997a): Differential modulation of the uptake currents by redox interconversion of cysteine residues in the human neuronal glutamate transporter EAAC1. *Eur. J. Neurosci.*, **9**, 2207-2212.
- Trotti, D., Rizzini, B.L., Rossi, D., Haugeto, O., Racagni, G., Danbolt, N.C. and Volterra, A. (1997b): Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. *Eur. J. Neurosci.*, **9**, 1236-1243.
- Wagner, C., Sudati, J.H., Nogueira, C.W. and Rocha, J.B. (2010): *In vivo* and *in vitro* inhibition of mice thioredoxin reductase by methylmercury. *Biometals*, **23**, 1171-1177.
- Yasutake, A., Nakano, A., Miyamoto, K. and Eto, K. (1997): Chronic effects of methylmercury in rats. I. Biochemical aspects. *Tohoku J. Exp. Med.*, **182**, 185-196.
- Yee, S. and Choi, B.H. (1994): Methylmercury poisoning induces oxidative stress in the mouse brain. *Exp. Mol. Pathol.*, **60**, 188-196.
- Yin, Z., Milatovic, D., Aschner, J.L., Syversen, T., Rocha, J.B., Souza, D.O., Sidoryk, M., Albrecht, J. and Aschner, M. (2007): Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes. *Brain Res.*, **1131**, 1-10.
- Yonaha, M., Saito, M. and Sagai, M. (1983): Stimulation of lipid peroxidation by methyl mercury in rats. *Life Sci.*, **32**, 1507-1514.
- Yoshida, E., Toyama, T., Shinkai, Y., Sawa, T., Akaike, T. and Kumagai, Y. (2011): Detoxification of methylmercury by hydrogen sulfide-producing enzyme in mammalian cells. *Chem. Res. Toxicol.*, **24**, 1633-1635.
- Yoshino, Y., Mozai, T. and Nakao, K. (1966): Biochemical changes in the brain in rats poisoned with an alkylmercury compound, with special reference to the inhibition of protein synthesis in brain cortex slices. *J. Neurochem.*, **13**, 1223-1230.
- Vogel, D.G., Margolis, R.L. and Mottet, N.K. (1985): The effects of methyl mercury binding to microtubules. *Toxicol. Appl. Pharmacol.*, **80**, 473-486.
- Vogel, D.G., Margolis, R.L. and Mottet, N.K. (1989): Analysis of methyl mercury binding sites on tubulin subunits and microtubules. *Pharmacol. Toxicol.*, **64**, 196-201.
- Zhang, Y., Lu, R., Liu, W., Wu, Y., Qian, H., Zhao, X., Wang, S., Xing, G., Yu, F. and Aschner, M. (2013): Hormetic effects of acute methylmercury exposure on grp78 expression in rat brain cortex. *Dose Response*, **11**, 109-120.