Tohoku J. Exp. Med., 2002, **196**, 65–70

Review

Investigation of Intracellular Factors Involved in Methylmercury Toxicity

Akira N, Takemitsu Furuchi, Nobuhiko Miura,
Gi-Wook Hwang and Shusuke Kuge

Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980–8578

Naganuma, A., Furuchi, T., Miura, N., Hwang, G.-W. and Kuge, S. *Investigation of Intracellular Factors Involved Methylmercury Toxicity.* Tohoku J. Exp. Med., 2002, **196** (2), 65–70 —— Methylmercury is a known pollutant that causes severe central nervous system disorders. It is capable of passing through the blood-brain barrier and accumulates in cerebral cells. However, little is known regarding the mechanism of its toxicity at the molecular level. Using yeast cells, we searched for the genes involved in the expression of methylmercury toxicity, and found that genes encoding L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT) and ubiquitin transferase (Ubc3) confer methylmercury resistance on the cells. It has also been shown that GFAT is the target molecule of methylmercury in yeast cells. These findings provide important clues about the mechanism underlying methylmercury toxicity in mammals. ———— methylmercury; resistance; yeast; ubiquitin; glucosamine-6-phosphate

The mechanism underlying mercury poisoning remains to be elucidated

Despite the efforts of many researchers, including ourselves, the mechanisms involved in methylmercury toxicity and the defense against this toxicity remain unknown (Akagi and Naganuma 2000; Miura 2000). Although more than 40 years have passed since the first outbreak of Minamata disease, we still only know that the causative agent is methylmercury which is highly cytotoxic, but how it exerts it toxicity at the molecular level remains to be determined. We have attempted to clarify the mechanism of methylmercury toxicity using experimental animals, because we consider it important to investigate the in vivo toxicity in detail, since studies using only in vitro experimental systems did not clarify the mechanism of the neurotoxicity of methylmercury. However, studies using rats and mice would be meaningless unless findings significant and relevant to

Received December 12, 2001; revision accepted for publication February 28, 2002.
Address for reprints: Akira Naganuma, Ph.D., Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980–8578, Japan.
e-mail: naganuma@mail.pharm.tohoku.ac.jp

This paper was presented at the Sendai Symposium: Paradigm Shift in Heavy Metal Toxicology held on November 21–22, 2001.

humans are obtained. We have obtained several toxicologically important findings with respect to methylmercury using experimental animals (Naganuma and Imura 1979, 1980, 1984; Naganuma et al. 1980a; Tanaka et al. 1991, 1992a, b; Urano et al. 1997), but could not determine the mechanism of methylmercury toxicity at the molecular level.

Does methylmercury target specific molecules in cells ?

By what mechanism does methylmercury exert it toxic effects on cells ? Methylmercury has a high affinity to the SH residues of cysteine, and inhibits most of the enzymes whose activities depend on the SH residues in the enzyme molecules. Therefore, some researchers consider that methylmercury does not exert its toxicity by attacking specific targets in cells but suppresses the growth of cells by the nonspecific inhibition of the activities of proteins with SH residues. In fact, increases in the concentration of intracellular free SH residues reduce methylmercury toxicity (Miura et al. 1994a, b). On the other hand, it was reported that methylmercury toxicity was suppressed by antioxidants such as vitamin E in animal experiments (Yonaha et al. 1983; Sarafian and Verity 1991), but this cannot be explained by the nonspecific inhibition of intracellular active substances by methylmercury.

Investigation of target molecules using yeast

If it is assumed that there are target molecules of methylmercury in cells, the problem of finding them remains. Many researchers have attempted to identify such target molecules, but most tested candidates among known intracellular factors. However, unknown factors cannot be identified by such a method. In this study, we randomly tested target molecules at the genetic level. We focused on the fact that drug resistance is sometimes involved in elevation of the concentration of the intracellular target of the drug. Screening for the genes conferring methylmercury resistance on cells transformed with individual genes would indicate the genes encoding the target molecules of methylmercury together with the genes for the defense against its toxicity. We investigated yeast genes obtained from a library, since yeast genes can be easily identified because the nucleotide sequence of the entire genome has been clarified. Plasmids carrying a chromosome fragment (usually containing 2–4 genes) were transfected into yeast cells, and genes contained in the chromosome fragments were expressed at high levels in the cells. Among such yeast cells, those that could grow on a medium containing methylmercury at a concentration that would not permit the growth of normal yeast cells were selected. Since these yeast cells acquired methylmercury resistance following the introduction of gene fragments, genes conferring methylmercury resistance must be contained in the introduced gene fragments. Plasmids were isolated from the yeast cells that had acquired

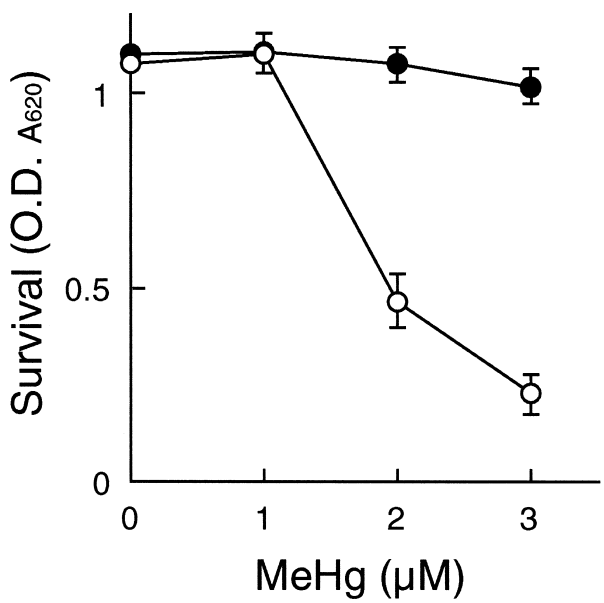

Fig. 1. Sensitivity of yeast transfected with the *GFAT* gene to toxicity of methylmercury (MeHg). W303B/pGFAT and W303B/pYES2 (control) were suspended in a medium in the presence of MeHg and cultured with shaking at 30°C for 24 hours.
○, pYES2 (Control); ●, GFAT.

methylmercury resistance, and the chromosome fragments carried in the plasmids were investigated. We identified *GFA1* (Fig. 1) and *CDC34* as the genes involved in methylmercury resistance (Miura et al. 1999; Naganuma et al. 2000; Furuchi et al. 2002; Hwang et al. 2002). *GFA1* is the gene encoding L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT), which is a catalytic enzyme involved in the production of glucosamine-6-phosphate from glutamine and fructose. *CDC34* is the gene encoding ubiquitin transferase (Ubc3), which is involved in the ubiquitination of intracellular proteins.

GFAT is the target molecule of methylmercury in yeast

Glucosamine-6-phosphate generated by the reaction catalyzed by GFAT is the precursor of all amino sugars synthesized intracellularly. Yeast cells cannot survive without amino sugars, because glycoproteins cannot be produced in their absence. Therefore, GFAT is an the essential enzyme for the growth of yeast cells (Watzele and Tanner 1989). Since GFAT is an SH enzyme, methylmercury inhibits GFAT activity. The inhibitory effects of methylmercury on various SH enzymes were determined (Naganuma et al. 2000) (Fig. 2). The activity of GFAT was almost completely inhibited by 4 μM methylmercury, while those of other SH enzymes were hardly affected by methylmercury at this concentration. The inhibition constant (Ki) of methylmercury was 4 μM for GFAT, while the Ki values for other SH enzymes were higher than 10-fold this value. These results indicate that methylmercury has a high affinity for GFAT and specifically inhibits its activity, suggesting that GFAT is the target molecule of methylmercury.

Yeast cells transfected with the *GFA1* gene are highly resistant to methylmercury, and a

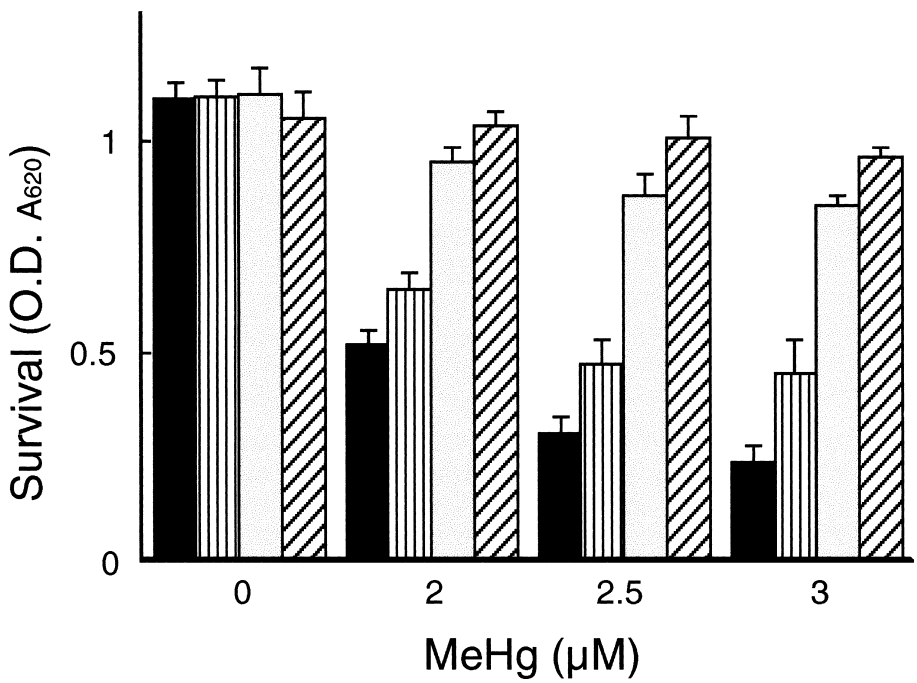

Fig. 2. Effect of MeHg on activities of GFAT and other SH-enzymes in extract of wild-type yeast (W303B). After a 3-minute incubation in yeast extract with MeHg at 37°C, the activities of GFAT, alcohol dehydrogenase (ADH), glutathione reductase (GR) and lactate dehydrogenase (LDH) were determined.
GlcN (mM) ■, 0; ▥, 6.25; □, 12.5; ▨, 25.

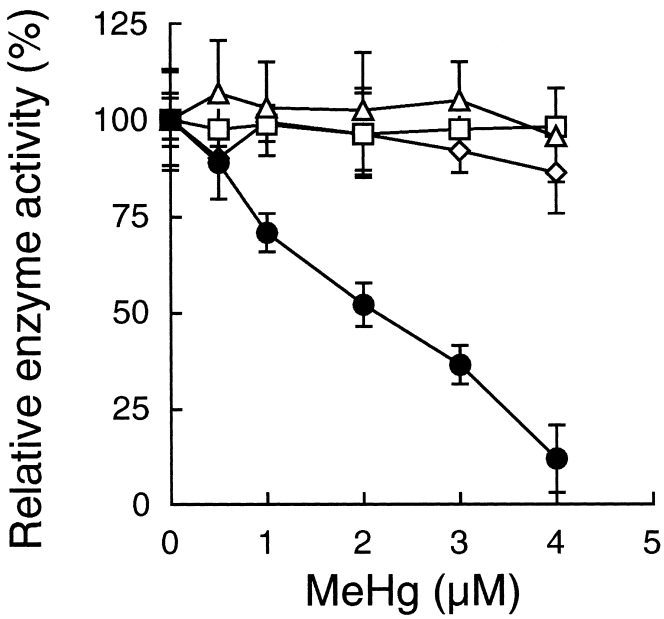

Fig. 3. Effect of an increase in intracellular levels of glucosamine-6-phosphate on toxicity of MeHg. Wild-type yeast (W303B) cells suspended in the medium were pretreated with glucosamine, which is converted to glucosamine-6-phosphate by hexokinase in yeast, for 1 hour followed by a 24-hour incubation with MeHg.
●, GFAT; △, ADH; □, GSHR; ◇, LDH.

relatively large amount of GFAT is synthesized by the cells. Therefore, it remains possible that strong binding of GFAT at a high concentration to methylmercury reduced the concentration of free methylmercury and suppressed methylmercury toxicity, inducing methylmercury resistance in the yeast cells. Therefore, we determined the effects of increasing the intracellular concentration of glucosamine-6-phosphate induced by the reaction catalyzed by GFAT on methylrercury toxicity. Since glucosamine-6-phosphoric acid added to media is not taken up by cells, glucosamine was added to media. Glucosamine is not synthesized by cells, but extracellularly added glucosamine is taken up by cells and transformed to glucosamine-6-phosphate by hexokinase. The toxicity of methylmercury towards the yeast cells was markedly reduced depending on the concentration of added glucosamine (Naganuma et al. 2000) (Fig. 3).

In conclusion, (1) yeast cells with high-level GFAT expression are resistant to methylmercury, (2) methylmercury specifically inhibits GFAT activity, (3) methylmercury toxicity is markedly reduced by the addition of glucosamine, which is transformed to glucosamine-6-phosphate, the product of the GFAT reaction, to cells, and (4) GFAT is an essential enzyme in yeast. These results suggest that GFAT is the main target molecule of methylmercury in yeast (Naganuma et al. 2000).

Ubiquitination as a defense mechanism against methylmercury toxicity

As described above, we showed that the gene encoding Ubc3, in addition to that encoding GFAT, confers methylmercury resistance on yeast. Ubc3 is an important enzyme in the ubiquitination of intracellular proteins. The ubiquitin system, which consists of a ubiquitin activation enzyme (E1), ubiquitin transferase (E2) and ubiquitin ligase (E3), is involved in the degradation of abnormal intracellular proteins. In this system, ubiquitin is activated by E1 and then binds to E2, while E3 recognizes target

proteins such as abnormal proteins. E2 bound to ubiquitin binds to E3, and transfers ubiquitin to the target protein. Finally, the target protein ubiquitinated by these reactions is recognized by proteasomes and rapidly degraded (Hochstrasser 1996; Hershko and Ciechanover 1998).

E2 proteins belong to the family of ubiquitin transferases, and the UBC domain is preserved as the catalytic domain in all E2 proteins. It is known that cysteine residues involved in the binding to ubiquitin, which is essential to the expression of E2 activity, are present in the UBC domain. To clarify the mechanism by which Ubc3 confers methylmercury resistance on yeast cells, we produced yeast cells overexpressing a mutant Ubc3 by substituting this cysteine residue with alanine, and found that these yeast cells were not methylmercury-resistant (Hwang et al. 2002). Therefore, the ubiquitin transfer activity of Ubc3 is considered essential to the acquisition of methylmercury resistance.

Thirteen enzymes of the E2 family of yeast have been identified, each considered to exhibit substrate specificity. Therefore, we produced yeast cells overexpressing Ubc2, Ubc4, Ubc5 or Ubc7 of the E2 family, and determined their methylmercury resistance. Methylmercury resistance was observed in the yeast cells overexpressing Ubc4, Ubc5 and Ubc7 (Furuchi et al. 2002). Our study was the first to show that high-level expression of E2 family enzymes confers resistance to toxic chemicals. Among the yeast cells overexpressing E1 (Uba1) or E3 (CDC53, SKP1, HRT1) proteins, only the yeast cells overexpressing Uba1 of the E1 family exhibited weak methylmercury resistance (Hwang et al. 2002). These results suggest that E2 is the rate-limiting enzyme in the ubiquitination reaction, and the amount of ubiquitinated protein within cells was markedly increased by the overexpression of Ubc3.

It is considered that protein denatured by active oxygen is ubiquitinated by the ubiquitin system and degraded. It is hypothesized that cytotoxicity results when abnormal proteins do not undergo normal degradation and accumulate in cells. Therefore, it is suggested that some modification of a specific protein within cells by methylmercury causes cytotoxicity, and enhancement of the degradation of the protein by ubiquitination reduces this toxicity. In this case, the ubiquitin system acts to protect cells against methylmercury toxicity, and the protein ubiquitinated following modification by methylmercury would be the target molecule of methylmercury toxicity.

There are genes in humans that are homologous to the genes encoding GFAT and Ubc3, which have been identified as proteins conferring methylmercury resistance on yeast cells. Therefore, both proteins are likely to be involved in methylmercury toxicity in human cells. To clarify the mechanisms of the toxicities of other toxic chemicals, investigation of resistance factors at the genetic level using yeast is considered useful.

References

Akagi, H. & Naganuma, A. (2000) Human exposure to mercury and the accumulation of methylmercury that is associated with gold mining in the Amazon basin, Brazil. *J. Health Sci*., **46**, 323–328.

Furuchi, T., Hwang, G.W. & Naganuma, A. (2002) Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methymercury in *Saccharomyces cerevisiae*. *Mol. Pharmacol.* (in press)

Hershko, A. & Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem*., **67**, 425–479.

Hochstrasser, M. (1996) Ubiquitination-dependent protein degradation. *Annu. Rev. Genet*., **30**, 405–439.

Hwang, G.W., Furuchi, T. & Naganuma, A. (2002) Aubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylemercury. *FASEB J.* (in press)

Miura, K. (2000) Methylmercury toxicity at cellular levels-from growth inhibition to apoptotic

cell death. *J. Health Sci*., **46**, 182–186.

Miura, K., Clarkson, T.W., Ikeda, K., Naganuma, A. & Imura, N. (1994a) Establishment and characterization of methylemercury-resistant PC12 cell line. *Environ. Health Perspect*., **102**, 313–315.

Miura, K., Ikeda, K., Naganuma, A. & Imura, N. (1994b) Important role of glutathione in susceptibility of mammalian cells to methylmercury. *In Vitro Toxicol*., **7**, 59–64.

Miura, N., Kaneko, S., Hosoya, S., Furuchi, T., Miura, K., Kuge, S. & Naganuma, A. (1999) Overexpression of L-glutamine: D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*. *FEBS Lett*., **458**, 215–218.

Naganuma, A. & Imura, N. (1979) Methylmercury binds to a low molecular weight substance in rabbit and human erythrocytes. *Toxicol. Appl. Pharmacol*., **47**, 613–616.

Naganuma, A. & Imura, N. (1980) Bis (methylmercuric) selenide as a reaction product from methylmercury and selenite in rabbit blood. *Res. Commun. Chem. Pathol. Pharmacol*., **27**, 163–173.

Naganuma, A. & Imura, N. (1984) Species difference in biliary excretion of methylmercury. *Biochem. Pharmacol*., **33**, 679–682.

Naganuma, A., Kojima, Y. & Imura, N. (1980a) Interaction of methylmercury and selenium in mouse: Formation and decomposition of bis (methylmercuric) selenide. *Res. Commun. Chem. Pathol. Pharmacol*., **30**, 301–315.

Naganuma, A., Kojima, Y. & Imura, N. (1980b) Behavior of methylmercury in mammalian erythrocytes. *Toxicol. Appl. Pharmacol*., **54**, 405–410.

Naganuma, A., Miura, N., Kaneko, S., Mishina, T., Hosoya, S., Miyairi, S., Furuchi, T. & Kuge, S. (2000) GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*. *FASEB J*., **14**, 968–972.

Naganuma, A., Nakajima, E., Shigehara, E., Tanaka, M. & Imura, N. (1983) Mercury distribution in mouse brain after iv administration bis (methylmercuric) selenide. *Toxicol. Lett*., **15**, 175–170.

Sarafian, T. & Verity, M.A. (1991) Oxidative mechanisms underlying methylmercury neurotoxicity. *Int. J. Dev. Neurosci*., **9**, 147–153.

Tanaka, T., Naganuma, A., Kobayashi, K. & Imura, N. (1991) An explanation for strain and sex differences in renal uptake of methylmercury in mice. *Toxicology*, **69**, 317–329.

Tanaka, T., Naganuma, A. & Imura, N. (1992a) Routes for renal transport of methylercury in mice. *Eur. J. Pharmacol.,* **228**, 9–14.

Tanaka, T., Naganuma, A., Miura, N. & Imura, N. (1992b) Role of testosterone in γ-glutamyltranspeptidase dependent renal methylmercury uptake in mice. *Toxicol. Appl. Pharmacol.,* **112**, 58–63.

Urano, T., Imura, N. & Naganuma, A. (1997) Inhibitory effect of selenium on biliary secretion of methylmercury in rats. *Biochem. Biophys. Res. Commun.,* **239**, 862–867.

Watzele, G. & Tanner, W. (1989) Cloning of the glutamine: fructose-6-phosphate amidotransferase gene from yeast. Pheromonal regulation of its transcription. *J. Biol. Chem.,* **264**, 8753–8758.

Yonaha, M., Saito, M. & Sagai, M. (1983) Stimulation of lipid peroxidation by methylmercury in rats. *Life Sci*., **32**, 1507–1514.