Bull. Environ. Contam. Toxicol. (2006) 77:779–784

DOI: 10.1007/s00128-006-1131-7

Bulletin of Environmental Contamination and Toxicology

Effects of Sodium Selenite on Methylmercury-Induced Cell Death and on Mercury Accumulation in Rat Cerebellar Neurons in Primary Culture

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Received: 27 July 2006/Accepted: 18 October 2006

Selenium, as selenite, an essential trace element (Schwarz and Foltz, 1957), is widely accepted as a substance that has protective effects against neurotoxicity of methylmercury by excessive supplementation in diets (Ganther et al., 1972; Potter and Matrone, 1974) or by simultaneous administration in experimental animals (Iwata et al., 1973; Ohi et al., 1975). Also in the case of *in vitro* neuronal culture systems, selenite protects neurons from methylmercury toxicity. Investigators indicate that methylmercury-induced cytotoxicity in cultured cerebral neurons and cerebellar granule cells from rodents is inhibited by selenite treatment (Park et al., 1996; Kasuya, 1976; Sakaue et al., 2005a), which suggests that selenite may have the ability to directly protect neurons against the cytotoxicity of methylmercury. Previous reports suggested that the protective effect of selenite results from acting an essential role in the activity of glutathione peroxidase because methylmercury treatment generates reactive oxygen species in the cells, and from conversing methylmercury to a less toxic form or a less toxic product by selenium and cysteine (Ganther, 1980; Hirata and Takahashi, 1981) or by directly forming a complex between selenium and methylmercury (Naganuma and Imura, 1980a; Naganuma et al., 1980b; Masukawa et al., 1982). Further, other investigators revealed that selenium treatment alters mercury uptake of cells in human erythroleukemia cell (Frisk et al., 2001) and rat erythrocytes (Yamane et al., 1977). There is little information regarding the protective roles of selenite against methylmercury toxicity in neurons, especially with regard to the effect of selenite on the uptake of methylmercury into neurons, although neurons are more sensitive to methylmercury than cells in other tissues.

Cerebellar granule cells, which are particularly vulnerable to methylmercury-induced damage *in vivo*, have provided a good model for the analysis of methylmercury-induced neuronal cell death and for determining effects of methylmercury on neuronal cells directly. Previous reports using the *in vitro* culture system of cerebellar neurons have demonstrated that methylmercury can induce cell death at low levels (Sakaue et al., 2003; Sakaue et al., 2005b). In the present study, we show that selenite treatment inhibits the uptake of methylmercury in cultured cerebellar neurons. Furthermore, we discuss the involvement of the methylmercury-uptake inhibition in the protective effect of selenite on neuronal cell death induced by methylmercury.

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MATERIALS AND METHODS

Primary neuron-rich cerebellar cell cultures were prepared from Wistar rats (Jcl:Wistar; Clea Co., Tokyo, Japan) within 24 hrs after birth, as described in previous reports (Sakaue et al., 2003; 2005b). Cerebella incubated with trypsin for 13 min at room temperature were minced by mild trituration with a Pasteur pipette. Cerebellar neurons were seeded in Eagle's minimal essential medium (Gibco BRL, Grand Island, NY) containing 1 mg/ml bovine serum albumin (BSA), 10 μg/ml bovine insulin, 0.1 nM thyroxine, 0.1 mg/mg human transferrin, 1 μg/ml aprotinin, 30 nM Na_2SeO_3, 0.25 % glucose, 100 units /mL penicillin, and 135 μg/mL streptomycin on poly-L-lysine-coated dishes and cultured for 2 days. All cell culture supplements were purchased from Sigma Chemical Company (St. Louis, MO). Then, cerebellar cell cultures were treated with methylmercuric chloride (Tokyo Kasei Kogyo. Co., Ltd., Tokyo, Japan) at 30 nM for 24 or 48 hrs with or without sodium selenite (Wako Pure Chemical Ind., Ltd., Osaka, Japan) at several doses. It is notable that the culture medium of all experiments contained sodium selenite at 30 nM because it is an essential trace element, as described above, even in the case of non-treatment with sodium selenite. The number of viable cerebellar cells in the culture was estimated by crystal violet staining, as described in previous reports (Sakaue et al., 2003; 2005b). All preparations of the primary cultures were done in accordance with the Kitasato University Guidelines for Animal Care and Experimentation.

Cerebellar cell cultures treated with methylmercury and sodium selenite for 24 hrs under the conditions described above were rinsed 3 times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS, pH 7.4). The cerebellar cell cultures were harvested in 0.1 % sodium dodecylsulfate (SDS) in PBS. Total mercury content in the samples was determined by the oxygen combustion-gold amalgamation method using a Rigaku Mercury Analyzer MA-2 (Nippon Instruments Co., Tokyo, Japan). Whole protein content was determined with a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.

All values are presented as means ± SEM. Statistical differences between the control group and treatment groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's test.

RESULTS AND DISCUSSION

To detect a relationship between methylmercury-induced cell death and the mercury content in cerebellar cell culture, we assayed cell viability and mercury content after methylmercury treatment at several dosages. The cell viability in the cerebellar cell culture 48 hrs after methylmercury treatment was significantly decreased in a methylmercury dose-dependent manner (Fig. 1a). At 3 nM, 10 nM, 30 nM, and 100 nM of methylmercury, the cell viabilities compared to the control were 99.9 ± 0.25, 94.8 ± 1.9, 66.3 ± 1.3 and 43.6 ± 0.8 percent, respectively (Fig. 1a). In contrast to cell viability, the mercury contents in the cerebellar cell cultures increased in a methylmercury dose-dependent manner. The mercury content values were 25.1 ± 3.3 to 542.0 ± 53.0 ng/mg protein 24 hrs after methylmercury

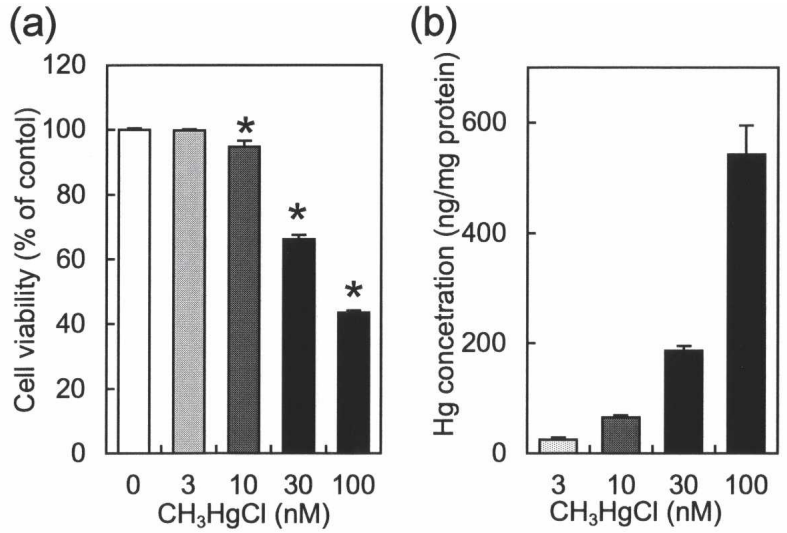

Figure 1. Methylmercury-induced cytotoxicity and mercury content on primary cerebellar cells. (a) Methylmercury-induced cerebellar cell death in a dose-dependent manner. The cells were fixed 48 hrs after methylmercury treatment, and elucidated for the cell viability using the crystal violet method. The data are shown as the mean ± SEM using cell viability in the cells treated with vehicle alone as a control (n=4). Statistically significant differences from the mean of the control group were analyzed by ANOVA followed by Dunnett's test (*, $p<0.05$). (b) The relationship between methylmercury concentration in the culture medium and mercury content in cultured cerebellar cells. The cells were harvested 24 hrs after methylmercury treatment. The data are shown as the mean ± SEM, n=3.

treatment at 3 to 100 nM (Fig. 1b). The cell viability at 48 hrs was decreased with a mercury content increasing at 24 hrs, which suggest that the cell death at 48 hrs is closely dependent to the mercury content in the cultures at 24 hrs.

To determine the effect of selenite on the cell death by methylmercury treatment, we assayed the cell viability of cerebellar cell culture 48 hrs after co-treatment of 30 nM methylmercury and selenite using the crystal violet staining method (Fig. 2a). The viability of the cerebellar neurons decreased 48 hrs after exposure to methylmercury at 30 nM, as described in previous studies (Sakaue et al., 2003; 2005a; 2005b). The decrement of cell viability on the cerebellar cell cultures exposed to 30 nM methylmercury was significantly restored by treatment with sodium selenite in a dose-dependent manner (Fig. 2a). The methylmercury affect on the cell viability has no change at 30 nM selenite, while sodium selenite treatment at 100 nM, 300 nM, and more, contributed to the partial and complete recovery of cell viability, respectively. Furthermore, to determine whether the inhibitory effect of selenite on the methylmercury-induced cell death as shown in Fig. 2a is changed by alterations of methylmercury uptake into the cells, we studied the effect of selenite on the mercury content in cerebellar cell cultures treated with 30 nM methylmercury, the level at which cells die after 48 hr of exposure. The total mercury content decreased with increasing sodium selenite concentration, and the decrement was statistically significant at 10 μM of sodium

selenite, 84.4 ± 0.7 % of the value at 0 nM selenite 24 hr after sodium selenite treatment (Fig. 2b). Only selenite treatment at 10 μM did not affect the cell viability after incubation for 24 hrs (data not shown).

It is well known that selenite inhibits methylmercury-induced neurotoxicity *in vivo* (Ganther et al., 1972; Potter and Matrone, 1974; Iwata et al., 1973; Ohi et al., 1975) and cell death of cerebellar neurons *in vitro* (Park et al., 1996; Kasuya, 1976; Sakaue et al., 2005a). However, there is little information about the mechanisms of the selenite protective effect. In the present study, we found that the mercury content in methylmercury-exposed cells decreased due to selenite treatment in a cerebellum primary culture system. This is the first report that indicates a decrement of mercury content in methylmercury-exposed neural cells through selenite treatment *in vitro*.

Mercury content in the brains of rat pups from mothers supplemented with dietary selenite alone was lower than in those from non-treatment mothers (Fredriksson et al., 1993). The offspring of pregnant mice co-administered methylmercury and selenite have a lower brain mercury content than that of those administered

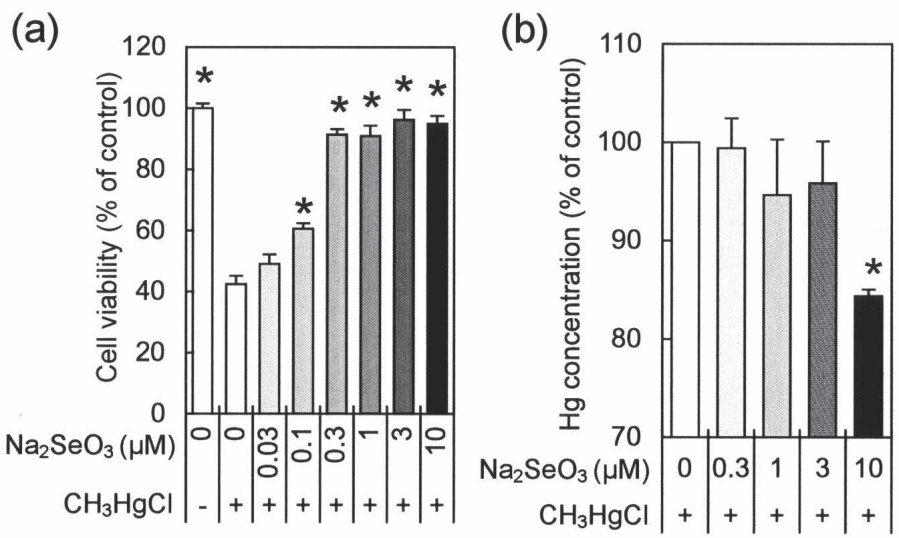

Figure 2. Inhibitory effect of sodium selenite on methylmercury-induced cytotoxicity and mercury content of primary cerebellar cells. (a) Suppression of the methylmercury-induced cell death via sodium selenite treatment. The cells were fixed 48 hrs after 30 nM methylmercury exposure and assayed using the crystal violet method to estimate the cell viability. The data is shown as the mean ± SEM using cell viability in the cells treated with vehicle alone as a control (n = 4). Asterisks in the graph indicate significant differences from the cells treated with methylmercury alone at $p<0.05$. (b) Alteration of mercury content of the cells by sodium selenite treatment. The cells were harvested 24 hrs after methylmercury and sodium selenite treatment. The data are showed as mean ± SEM using Hg levels in the cells treated with methylmercury alone as a control (n=3). Asterisks in the graph indicate statistical significance as compared to the group treated with only methylmercury ($p<0.05$).

methylmercury alone (Satoh et al., 1985a; 1985b). Moreover, there are reports of decreased mercury content in a human erythroleukemia cell line, K-526, with increased selenite (Frisk et al., 2001) and that selenite administration to rats inhibits mercury uptake by rat erythrocytes (Yamane et al., 1977). Those reports are in agreement with the result in the present study as regards the decrement of mercury content through selenite treatment, which might be caused by directly forming a complex between selenium and methylmercury in the culture medium (Naganuma and Imura, 1980a; Naganuma et al., 1980b; Masukawa et al., 1982). Methylmercury binds cysteine to form the cysteine S-conjugate, which is similar structurally to the amino acid methionine. System L, a major transporter of neutral amino acid, transports methionine into cytosol of cultured calf brain capillary endothelial cells and also cysteine S-conjugate of methylmercury (Mokrzan et al., 1995). Two isoforms of system L, L type large neutral amino acid transporters 1 and 2, are expressed in astrocytes and endothelial cells, and also in primary culture of neurons (Heckel et al., 2003). In this case, the formation of a complex of selenite and methylmercury in the extra-cellular region may affect the methylmercury uptake into neurons via the transporter. Further studies are essential to clarify the effect of the formation of methylmercury-selenite complex by selenite treatment on the uptake of methylmercury into the cells as methylmercury cysteine S-conjugate.

As shown in Fig. 2, an inhibitory effect of selenite on methylmercury-induced cell death was detected at lower concentrations of selenite than the concentration at which mercury content in the cells decreased. These results suggest that selenite treatment have protective effects against cell death without decreasing mercury accumulation in the cells *in vitro*, and the selenite protection effect may contain any mechanisms of the protection other than the reduction of mercury content in the methylmercury-treated cultures. Supposedly, the formation of the methylmercury-selenite complex may account for the inhibition of methylmercury neurotoxicity at lower selenite concentrations. When the methylmercury-selenite complex forms in the cell, methylmercury cytotoxicity may decrease with no affection the mercury content of the cell like as the result in the present study. In any case, the intracellular formation of methylmecury-selenite complex remains to be unknown in this study. Further studies utilizing an *in vitro* culture system are needed to explore the mechanism of direct selenite protection of cells against methylmercury neurotoxicity containing intracellular amount of methylmercury-selenite complex.

*Acknowledgments.*The authors thank Ms. Rieko Ochiai for her technical assistance with mercury and protein assays in methylmercury-treated cells. This work was supported in part by a Kitasato University Research Grant for Young Researchers (to MS).

REFERENCES

Fredriksson A, Gardlund AT, Bergman K, Oskarsson A, Ohlin B, Danielsson B, Archer T (1993) Effects of maternal dietary supplementation with selenite on the postnatal

development of rat offspring exposed to methyl mercury in utero. Pharmacol Toxicol 72: 377-382.
Frisk P, Yaqob A, Nilsson K, Lindh U (2001) Selenite or selenomethionine interaction with methylmercury on uptake and toxicity showing a weak selenite protection: studies on cultured K-562 cells. Biol Trace Elem Res 80: 251-268.
Ganther HE (1980) Interactions of vitamin E and selenium with mercury and silver. Ann NY Acad Sci 355: 212-225.
Ganther HE, Goudie ML, Kopecky MJ, Wagner P, Oh SH, Hoekstra WG (1972) Selenium: relation to decreased toxicity of methylmercury added to diets containing tuna. Science 175: 1122-1124.
Hirata E, Takahashi H (1981) Degradation of methyl mercury glutathione by the pancreatic enzymes in bile. Toxicol Appl Pharm 578: 483-491.
Heckel T, Broer A, Wiesinger H, Lang F, Broer S (2003) Asymmetry of glutamine transporters in cultured neural cells. Neurochem Int 43: 289-298.
Iwata H, Okamoto H, Ohsawa Y (1973) Effect of selenium on methylmercury poisoning. Res Commun in Chem Pathol Pharmacol 5: 673-680.
Kasuya M (1976) Effect of selenium on the toxicity of methylmercury on nervous tissue in culture. Toxicol Appl Pharm 35: 11-20.
Masukawa T, Kito H, Hayashi M, Iwata H (1982) Formation and possible role of bis(methylmercuric)selenide in rats treated with methylmercury and selenite. Biochem Pharmacol 31: 75-78.
Mokrzan EM, Kerper LE, Ballatori N, Clarkson TW (1995) Methylmercury-thiol uptake into cultured brain capillary endothelial cells on amino acid system L. J Pharmacol Exp Ther 272: 1277-1284.
Naganuma A, Imura N (1980a) Bis(methylmercuric)selenide as a reaction product from methylmercury and selenite in rabbit blood. Res Commun in Chem Pathol Pharmacol 27: 163-173.
Naganuma A, Kojima Y, Imura N (1980b) Interaction of methylmercury and selenium in mouse: formation and decomposition of bis(methylmercuric)selenide. Res Commun in Chem Pathol Pharmacol 30: 301-316.
Ohi G, Seki H, Maeda H, Yagyu H (1975) Protective effect of selenite against methylmercury toxicity: Observations concerning time, dose, and route factors in the development of selenium attenuation. Ind Health 13: 93-99.
Park ST, Lim KT, Chung YT, Kim SU (1996) Methylmercury-induced neurotoxicity in cerebral neuron culture is blocked by antioxidants and NMDA receptor antagonists. Neurotoxicology 17: 37-46.
Potter S, Matrone G (1974) Effect of selenite on the toxicity of dietary methyl mercury and mercuric chloride in the rat. J Nutr 104: 638-647.
Sakaue M, Takanaga H, Adachi T, Hara S, Kunimoto M (2003) Selective disappearance of an axonal protein, 440-kDa ankyrin$_B$, associated with neuronal degeneration induced by methylmercury. J Neurosci Res 73: 831-839.
Sakaue M, Mori N, Okazaki M, Kawakami S, Ikeda M, Nakamura R, Hara S, Sakabe K (2005a) Effect of culture conditions on the methylmercury neurotoxicity in methylmercury-induced cell death model using primary neuronal culture from cerebellum of neonatal rat. Japanese J. Clin. Environ. (in Japanese) 14: 106-111.
Sakaue M, Okazaki M, Hara S (2005b) Very low levels of methylmercury induce cell death of cultured rat cerebellar neurons via calpain activation. Toxicology 213: 97-106.
Satoh H, Shimai S, Yasuda N (1985a) Mercury metabolism and development of offspring perinatally exposed to methylmercury and selenite. Nutr Res Suppl. 1: 580-586.
Satoh H, Yasuda N, Shimai S (1985b) Development for reflexes in neonatal mice prenatally exposed to methylmercury and selenite. Toxicol Lett 25: 199-203.
Schwarz K, Foltz CM (1957) Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J American Chem Soc 79: 3292-3293.
Yamane Y, Fukino H, Aida Y, Imagawa M (1977) Studies on the mechanism of protective effects of selenium against the toxicity of methylmercury. Chem Pharm Bull 25: 2831-2837.