L-Glutamate Enhances Methylmercury Toxicity by Synergistically Increasing Oxidative Stress

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Abstract. Methylmercury (MeHg) is a well-known environmental toxicant. With its lipophilic nature and high reactivity to sulfhydryl groups, it is widely distributed and accumulated in the body to damage cells. Oxidative stress is proposed as a major mechanism underlying the cytotoxic action of MeHg. In the present study, we found that L-glutamate (L-Glu) concentration-dependently increased MeHg cytotoxicity in HeLa S3 cells. The enhancement of the toxicity was accompanied by enhanced apoptosis, increased production of reactive oxygen species, and decreased glutathione level. An anti-oxidant N-acetylcysteine largely alleviated the cytotoxicity, suggesting enhanced oxidative stress behind L-Glu-elicited increase of MeHg toxicity. The effect was specific to L-Glu and L-α-aminoadipate, whereas D-Glu, L-aspartate, and D-aspartate were not effective. In addition, the cystine uptake by the cells was mostly mediated by a L-Glu/L-α-aminoadipate–sensitive amino acid transport system xC. All these results suggest that the inhibition of system xC by L-Glu underlies the enhancement of MeHg cytotoxicity. The enhancement was highly synergistic because MeHg and L-Glu alone had little toxic effect in the conditions used. This synergism was confirmed in neural cells (neuroblastoma cell lines). It is proposed that similar mechanisms may underlie the neural toxicity of MeHg, particularly in the locality of lesions characteristic of MeHg toxicity.

Keywords: methylmercury, L-glutamate, cytotoxicity, apoptosis, oxidative stress

Introduction

Methylmercury (MeHg) is a well-known environmental pollutant that continues to pose great risks to human health. The lipophilic nature of MeHg facilitates its distribution throughout the body. Intracellularly, MeHg binds to a sulfhydryl group (–SH) and may bind to a variety of enzyme systems including those of microsome and mitochondria, causing cell injury and cell death (1). Despite several mechanisms proposed for MeHg toxicity, the formation of reactive oxygen species (ROS) with the disruption of mitochondrial function is supposed to be the major mechanism of cell damage (2, 3). Increased ROS level by MeHg has been reported both in vitro and in vivo. For example, cultured neurons (4) and glial cells (5) exposed to MeHg and brain synaptosomes prepared from animals injected with MeHg (6) demonstrated the increase of ROS production. In humans, MeHg poisoning is characterized by damage to discrete anatomical areas of the brain such as the visual cortex and the granule layers of the cerebellum (7). These discrete areas of neuronal damage and mechanism of MeHg toxicity are not well understood.

L-Glutamate (L-Glu) functions as a major excitatory neurotransmitter in the brain. It has been demonstrated that L-Glu induces ROS production in the brain via two distinct mechanisms. The first is mediated by glutamate receptors. Overstimulation of glutamate receptors causes
injury or death of neurons by a mechanism termed excitotoxicity (8, 9). ROS production, as well as mitochondrial Ca$^{2+}$ overload, mitochondrial depolarization and ATP depletion, is one of the important mechanisms underlying the excitotoxic damage of neurons (10, 11). The second mechanism of L-Glu-induced ROS production is via the inhibition of system $x^-_C$. System $x^-_C$ is the cystine/glutamate exchanger that mediates the uptake of cystine in exchange for $L$-Glu. It plays an important role to provide cells with cystine for glutathione (GSH) synthesis (12, 13). $L$-Glu inhibits cystine uptake of the cells by the inhibition of system $x^-_C$ in a competitive manner that decreases intracellular GSH level and, in turn, increases ROS (13, 14).

Besides its permeation through the plasma membrane by simple diffusion, MeHg is transported into cells by system L amino acid transporters when it is conjugated by cysteine (MeHg-Cys) (15). In the course of our study to examine which amino acids inhibit MeHg-Cys uptake in HeLa S3 cells by the competition at system L, we unexpectedly found that L-Glu greatly enhances the toxicity of MeHg in HeLa S3 cells. In the present study, to explore the mechanisms underlying the enhancement of MeHg toxicity by L-Glu, we have examined the effects of L-Glu on the MeHg-induced cell death, ROS production, and GSH level. We have found that L-Glu increases oxidative stress to induce apoptotic cell death probably due to the inhibition of system $x^-_C$.

**Materials and Methods**

**Materials**

All reagents and chemicals used were purchased from Sigma (St. Louis, MO, USA) unless specified otherwise. Methylmercury chloride was purchased from WAKO Pure Chemical Ind., Ltd. (Osaka).

**Cell culture**

HeLa S3 (human cervix adenocarcinoma) cells were maintained in Minimal Essential Medium (MEM) containing 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified 5% CO$_2$ incubator. Neuro2A (mouse neuroblastoma), NIE115 (mouse neuroblastoma), and NG108-15 (mouse neuroblastoma × rat glioma hybrid) cells were maintained in Dulbecco's Modified Eagle’s Medium (DMEM) with the same supplements and conditions described above (16–18). In some experiments, HeLa S3 cells were pretreated with 1 mM N-acetylcysteine (NAC) 12 h before the addition of MeHg and/or L-Glu. At the end of the incubation time, cell viability, the externalization of phosphatidylserine (PS), loss of mitochondrial membrane potential ($\Delta \Psi_{m}$), ROS, and GSH level were measured.

**Cytotoxicity assay**

MTT assay was used to assess cell viability. Cells were seeded onto 24-well plates at density of $1 \times 10^{4}$ cells/ml and cultured for 48 h before starting of the experiment. At the end of treatment, cell samples were incubated with 10% (v/v) stock MTT solution (5 mg/ml) for 4 h at 37°C. After incubation, a blue formazan crystal was solubilized with 0.04 M HCl in absolute isopropanol. Aliquots were quantitated spectrophotometrically at 570 nm by a UV/visible spectrophotometer (Ultrospec 2100; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Relative number of viable cells in each treatment condition to the untreated control (=100%) was calculated and presented in the Figs.

**Apoptosis assay using flow cytometry**

The externalization of phosphatidylserine (PS) during apoptosis was evaluated by fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) staining using an Annexin V–FITC Apoptosis Detection Kit from BD Biosciences (San Jose, CA, USA). After the indicated time, cells were harvested, washed twice with phosphate-buffered saline (PBS), and stained with Annexin V–FITC and PI according to manufacturer’s instruction. Cells were analyzed immediately with FACS Calibur (Becton Dickinson, Sunnyvale, CA, USA). Data were collected and analyzed using Cell Quest software (Becton Dickinson). To evaluate the $\Delta \Psi_{m}$, MitoProbe™DiIC1(5), a mitochondrial specific dye (Molecular Probes, Leiden, Netherlands) was used. The fluorescence intensity from the dye is reduced when mitochondrial membrane potential is disrupted (19). At the end of treatment, cells were incubated with 50 nM DiIC1(5) for 30 min at 37°C and analyzed using FACS Calibur. A minimum of 10,000 cells per sample was analyzed using Cell Quest software. As a positive control, cells were treated with 50 μM of the protonophore uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone.

**Measurement of intracellular ROS level using flow cytometry**

Intracellular ROS level was monitored by using the peroxide-sensitive fluorescence probe 2’,7’-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes). Cells were preloaded with 20 μM DCFH-DA for 15 min at 37°C in the dark before treatment. The fluorescent dye DCFH-DA passes through the cell membrane and undergoes deacetylation by intracellular esterases to produce the non-fluorescent compound.
DCFH that is trapped inside the cells. Oxidation of DCFH by ROS produces the highly fluorescent DCF. Hydrogen peroxide (250 μM) was used as a positive control for this measurement. Intracellular ROS levels were measured using FACS Calibur and analyzed using Cell Quest software.

**Intracellular GSH assay**

GSH was quantified by using a QuantiChrom™ Glutathione Assay Kit (Bioassay, Hayward, CA, USA). Buthionine-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, was used as a positive control of the reaction. The assay was performed according to the manufacturer’s instructions. Protein concentration was determined by the bicinchoninic acid (BCA) assay.

**Semiquantitative analysis of xCT mRNA expression**

Total cellular RNA was isolated and purified from cells by using an RNeasy Mini Kit according to the manufacturer’s instruction (Qiagen Sciences, MD, USA). The first-strand complementary DNA (cDNA) was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for xCT (20) and β-actin (internal control). PCR was performed by a GeneAmp (PCR system 9700; Applied Biosystems, Foster City, CA, USA) for 25 and 15 cycles with specific primers for human xCT (21) and human β-actin (22), respectively. The sense and antisense primers for human xCT were 5′-GTCAGAAAGCCTGTTGTGTCCACCA-3′ and 5′-TAAGAAAATCTGGATCGGGCCG-3′, corresponding to nucleotides 139–163 and 696–717 of human xCT (GenBank accession no. AB040875). The sense and antisense primers for human β-actin were 5′-CAAGAGATGGCCACGGCTGTCC-3′ and 5′-TCCTTCTGCATCTTGCGGCACGACG-3′ corresponding to nucleotides 2181–2202 and 2434–2455 of human β-actin (GenBank accession no. NM001101). The amount of PCR products were quantified by densitometric analysis and normalized in relation to the amount of β-actin mRNA. Visualization and densitometric analysis of band intensity of each sample was performed by a LAS-4000 digital imaging system (mini version 2.0) with Multi Gauge (version 3.1) software (Fuji Photo Film Co., Tokyo).

**Cystine uptake measurement**

HeLa S3 cells were seeded on 24-well plates (10^5 cells/well) and cultured for 48 h. Cells were then, incubated in MEM containing 10 mM L-Glu alone, 8 μM MeHg alone or MeHg plus L-Glu for 10 h. At the end of incubation, cells were washed three times with Na⁺-free Hank’s balance salt solution (HBSS) containing 125 mM choline-Cl, 25 mM HEPES, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, and 5.6 mM glucose (pH 7.4) and further incubated in the same solution at 37°C for 10 min. Cystine uptake by HeLa S3 cells was initiated by incubating the cells in Na⁺-free HBSS containing 5 μM [³¹C]L-cystine (PerkinElmer Life & Analytical Science, MA, USA) at 37°C for 5 min. Uptake was terminated by washing three times with ice-cold Na⁺-free HBSS. Then the cells were lysed with 0.1 N NaOH and the radioactivity was measured by an Aloka LSC-5100 β-scintillation counter (Aloka, Tokyo). An aliquot of cell lysate was used to determine protein concentration by the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

In order to examine the inhibitory effects of L-cystine, L-Glu, L-α-aminoacidopate, and L-Asp on cystine transport, the uptake of [³¹C]L-cystine (5 μM) by HeLa S3 cells was measured in the presence of 1 mM unlabeled L-cystine, L-Glu, L-α-aminoacidopate, or L-Asp. The cystine uptake in the presence of the inhibitors was expressed as percentage of control cystine uptake measured in the absence of inhibitors.

**Data and statistical analysis**

Data were presented as mean ± S.E.M. of three independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey’s post hoc analysis for multiple comparisons.

**Results**

**Enhancement of MeHg cytotoxicity by L-Glu in HeLa S3 cells**

Effect of L-Glu on MeHg toxicity was evaluated in HeLa S3 cells. The cells were treated with various concentrations of MeHg (2 to 8 μM) and MeHg plus 10 mM L-Glu. Cell viability was assessed at 3, 6, and 12 h from the start of the treatment. As depicted in Fig. 1A, cell viability was slightly decreased in cells treated with 8 μM MeHg alone at 12 h as compared to the control (P<0.05). No toxic effect was detected for lower concentration of MeHg alone at the time analyzed (Fig. 1A). Treatment with 4 and 8 μM of MeHg plus 10 mM L-Glu resulted in the markedly decreased cell viability compared to MeHg alone (P<0.05 and P<0.01, respectively) (Fig. 1A). The viability of cells treated with 8 μM MeHg together with L-Glu for 6 and 12 h were decreased by 35 ± 4% and 55 ± 5%, respectively (P<0.01, compared to MeHg alone). As shown in Fig. 1B, cytotoxicity was increased dependently on the concentration of L-Glu. By treatment with MeHg (8 μM)
**Fig. 1.** Effect of L-Glu on MeHg cytotoxicity in HeLa S3 cells. HeLa S3 cells were exposed to MeHg (2, 4, and 8 μM) or MeHg plus 10 mM L-Glu for 3, 6, and 12 h. At the end of the incubation period, cell viability was determined by MTT assay. A: the time course of the effect of MeHg or MeHg plus 10 mM L-Glu. The values were expressed as percentage of the untreated control [MeHg(−)]. L-Glu[−]). *P<0.05 vs control, †P<0.05 vs 4 μM MeHg alone, ‡P<0.01 vs 8 μM MeHg alone. B: concentration-dependence of L-Glu to enhance cytotoxicity of 8 μM MeHg at 12 h. Cell viability was measured with 8 μM MeHg [MeHg(+)] or without MeHg [MeHg(−)] at varied concentration of L-Glu (1 – 10 mM). The values were expressed as percentage of the untreated control [MeHg(−), L-Glu 0 mM]. P<0.05, †P<0.01.

plus L-Glu (1, 5, and 10 mM), the cell viability was significantly decreased when compared with MeHg alone, whereas L-Glu alone had no effect on cell viability at the concentration tested (Fig. 1B).

**Spectrum of amino acids to enhance MeHg-cytotoxicity**

To investigate whether the enhancement of MeHg toxicity was specific to L-Glu or not, twenty naturally occurring L-amino acids (Glu, Asp, Cys, Gly, Gln, Phe, His, Arg, Trp, Asn, Pro, Tyr, Lys, Ile, Val, Leu, Ser, Met, Ala, and Thr) at 10 mM were first examined on MeHg-induced cytotoxicity. Only L-Glu caused significant decrease in cell viability in the presence of 8 μM MeHg (data not shown). Then we examined Glu-related acidic amino acids D-Glu, L-Asp, D-Asp, and L-α-aminoadipate (L-AAD). As shown in Fig. 2, L-AAD was effective like L-Glu, whereas D-Glu, L-Asp, and D-Asp did not significantly alter MeHg toxicity.

**Effects of L-Glu on MeHg-induced apoptosis**

HeLa S3 cells treated with MeHg and/or L-Glu were stained with FITC-conjugated Annexin V and PI and analyzed by FACS. The different staining patterns depicted in Fig. 3A reflect different modes/stages of cell death: healthy cells (FITC−/PI−), early apoptotic cells (FITC+/PI−), late apoptotic cells (FITC+/PI+), and necrotic cells (FITC+/PI+). As shown in Fig. 3A, treatment with both MeHg (8 μM) and L-Glu (10 mM) markedly increased early and late apoptotic cells, whereas the increase in apoptosis was not evident for MeHg alone or L-Glu alone compared to the control. Then, whether the enhancement of MeHg-induced apoptosis by L-Glu was accompanied by alteration of ΔΨm was examined. As shown in Fig. 3B, mitochondrial membrane was markedly depolarized at 12 h when cells were treated with MeHg (8 μM) plus L-Glu (10 mM) as indicated by the significant decrease in DiIC1(5) fluorescence by 23 ± 2% (P<0.05, as compared to MeHg alone) (Fig. 3B). MeHg or L-Glu alone had no effect on ΔΨm.
Effects of L-Glu and MeHg on intracellular ROS and GSH

The level of ROS in the cells treated with MeHg (8 μM) and/or L-Glu (10 mM) was determined by staining the cells with the ROS sensitive dye DCFH-DA. As shown in Fig. 4A, MeHg alone increased the ROS level 4.7 ± 2-fold as compared to the control (P<0.01) at 12 h. MeHg with L-Glu had much greater effect on the ROS level than MeHg alone (52 ± 2-fold, P<0.01, compared to MeHg alone at 12 h). No significant effect was detected for L-Glu alone on the ROS level.

To determine if the increased ROS level following MeHg and L-Glu treatment was associated with the depletion of intracellular antioxidants, the intracellular GSH level was measured. Treatment of cells with BSO for 12 h as a positive control for GSH depletion decreased GSH levels to approximately 50% as compared to the untreated control (data not shown). As shown in Fig. 4B, L-Glu did not affect the GSH level. MeHg alone significantly decreased GSH level at 12 h by 28 ± 4% compared to the untreated control (P<0.05). GSH level was markedly decreased by 59 ± 6% by the co-treatment with MeHg and L-Glu at 12 h (P<0.05, as compared to MeHg alone). As shown in Fig. 4C, pretreatment with an
antioxidant, NAC, was effective in reducing the toxicity exhibited by MeHg plus L-Glu (P<0.01, as compared to MeHg plus L-Glu without NAC).

**System x⁻C cystine/glutamate exchanger in HeLa S3 cells**

The level of expression of cystine/glutamate exchanger xCT was assessed by semiquantitative PCR analysis. As shown in Fig. 5A, xCT mRNA was detected in untreated HeLa S3 cells and its level was increased by the treatment with MeHg (8 μM) and MeHg (8 μM) plus L-Glu (10 mM) for 6 h. Densitometric analysis of band intensities showed that the treatment with MeHg alone and MeHg plus L-Glu for 12 h significantly increased the level of xCT mRNA (2.1- and 2.4-fold as compared to the control, P<0.01, respectively) (Fig. 5B). L-Glu alone tended to increase xCT mRNA level at 12 h, although it was not statistically significant (Fig. 5B).

The activity of cystine/glutamate exchanger in HeLa S3 cells was also assessed by the measurement of [¹⁴C]l-cystine uptake (Fig. 5C). The [¹⁴C]l-cystine uptake of untreated HeLa S3 cells was inhibited by L-cystine, L-Glu, and L-AAD, but not by L-Asp (Fig. 5D). The [¹⁴C]l-cystine uptake activity was slightly increased by the treatment with L-Glu (10 mM) or MeHg (8 μM) and largely increased by the treatment with MeHg (8 μM) plus L-Glu (10 mM) (Fig. 5C). The increased L-cystine uptake was also inhibited by L-cystine, L-Glu, and L-AAD, but not by L-Asp, similar to that of untreated HeLa S3 cells (Fig. 5D).

**Enhancement of MeHg cytotoxicity by L-Glu in neuroblastoma cell lines**

To examine whether the enhanced MeHg toxicity by L-Glu is also applicable to neural cell lines, we investigated three neuroblastoma cell lines. Neuroblastoma cell lines Neuro2A, NIE115, and NG108-15 exhibited somewhat higher sensitivity to MeHg compared with HeLa S3 cells (data not shown). We used the maximum concentration of MeHg at which MeHg itself exhibited less effect on the viability of the cells (Fig. 6: A, B, and C). Consistent with the observation for HeLa S3 cells, L-Glu (10 mM) significantly reduced the viability of the cells when co-treated with MeHg, whereas L-Glu (10 mM) alone had no effect on the cell viability (Fig. 6: A, B, and C).

**Discussion**

In the present study, we showed that L-Glu unequivocally enhances MeHg toxicity. The enhancement of the toxicity is accompanied by enhanced apoptosis, increased ROS production, and decreased anti-oxidant system. An anti-oxidant NAC largely alleviated the cytotoxicity, suggesting that enhanced oxidative stress underlies the increase of toxicity of MeHg elicited by L-Glu. Furthermore, we revealed that, among amino acids...
tested, L-Glu and L-AAD are similarly effective, whereas D-Glu, L-Asp, and D-Asp were not effective in enhancing MeHg cytotoxicity. Cystine uptake of the cells was strongly inhibited by L-Glu and L-AAD but not by L-Asp. These characteristics are consistent with the involvement of the inhibition of amino acid transport system x^C in the enhancement of MeHg cytotoxicity by L-Glu. To our knowledge, this is the first study to demonstrate that the co-administration of MeHg and L-Glu results in increased cytotoxicity.

In this study, we used a non-neuron-derived cell line HeLa S3 to examine the mechanism underlying the L-Glu-induced enhancement of MeHg toxicity. This cell line was relatively resistant to MeHg toxicity, so that we were able to find easily that L-Glu greatly enhanced the toxicity of MeHg in the condition that MeHg exhibited little effect on the cell viability. We furthermore confirmed that this enhancement of MeHg toxicity by L-Glu was also applicable to neural cells such as neuroblastoma cell lines that showed somewhat higher sensitivity to MeHg than HeLa S3 cells. L-Glu concentration-dependently decreased the cell viability when the cells were exposed to MeHg in the condition that MeHg alone and L-Glu alone exhibited only a small effect or no effect on cell viability (Fig.1). In the presence of L-Glu, the toxic effect of MeHg was also
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dependent on the concentration of MeHg. These results indicate that L-Glu increases the sensitivity of the cells to MeHg toxicity.

Prevailing evidences suggest that ROS acts as a potent mediator of MeHg cytotoxicity (23). In this study, we confirmed that MeHg alone decreased cell viability at higher concentration for longer exposure time, which was accompanied by the increase of ROS and the decrease of GSH (Figs. 1 and 4). L-Glu drastically enhanced MeHg-induced increase of ROS and decrease of GSH. Furthermore, the cells were rescued from the enhanced toxicity by the treatment with the anti-oxidant NAC, suggesting that the enhanced oxidative stress underlies the enhanced cytotoxicity of MeHg co-treated with L-Glu.

Several studies have suggested that L-Glu is involved in MeHg toxicity in some different mechanisms. One possible mechanism is that MeHg inhibits cellular L-Glu uptake by inhibiting glutamate transporters, which elevates extracellular L-Glu concentration and induces cell death caused by overstimulation of glutamate receptors (excitotoxicity) (24, 25). Another possible mechanism is that MeHg selectively inhibits the uptake system for cystine and cysteine (26, 27) and thus compromises GSH synthesis, which would ultimately increase susceptibility of the cells to ROS produced by the stimulation of glutamate receptors (28). In these two mechanisms, neuronal cell death is caused by the excitotoxicity. In contrast, the third mechanism involves oxidative glutamate toxicity: elevated extracellular L-Glu causes prolonged cell death due to sustained oxidative stress resulting from the inhibition of amino acid transport system $x_c$ and the resultant decrease in GSH synthesis (29, 30).

System $x_c$ is the cystine/glutamate exchanger that mediates the cellular uptake of cystine in exchange for L-Glu (12, 13). Because the cytoplasm contains a high concentration of L-Glu, cystine uptake by system $x_c$ is driven by an outwardly directed electrochemical gradient of L-Glu via the exchange mechanism. Cystine uptake by system $x_c$ is, however, inhibited by L-Glu in a competitive manner, if L-Glu exists at high concentration at the cis-side (13). The binding site of system $x_c$ accepts cystine and L-form of acidic amino acids with longer side chains such as L-Glu and L-AAD, whereas acidic amino acids with shorter side chains such as L-Asp do not interact with system $x_c$ (14, 15). We found in the present study that L-Glu and L-AAD were effective, whereas D-Glu, L-Asp, and D-Asp were not effective in enhancing MeHg cytotoxicity (Fig. 2). Additionally, cystine uptake of both untreated cells and cells treated with MeHg and/or L-Glu was strongly inhibited by L-Glu and L-AAD but not by L-Asp, suggesting that background and induced cystine uptake of HeLa S3 cells was mainly due to system $x_c$ (14, 15). The involvement of glutamate receptor–mediated excitotoxicity in the enhancement of MeHg cytotoxicity seems less probable because the agonists of glutamate receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, and N-methyl-D-aspartate (NMDA) did not enhance the toxicity of 8 μM MeHg at concentrations of 10 – 100 μM, which are supposed to fully activate non-NMDA and NMDA receptors (S. Amonpatumrat et al., unpublished observation) (31, 32). It is, thus, proposed that the effect of
L-Glu to enhance MeHg cytotoxicity observed in this study is derived from the inhibition of system xC by L-Glu.

Because cysteine required for GSH synthesis is oxidized to cystine in the extracellular environment, system xC is important to provide cells with cysteine as cystine for GSH synthesis as already mentioned. Thus, the sustained inhibition of system xC by L-Glu makes the cells liable to the accumulation of ROS and ultimately leads to cell death by oxidative stress. Cells usually respond to the oxidative glutamate toxicity by enhancing apoptosis (29, 30). Consistent with this, we observed that L-Glu greatly increased MeHg-induced apoptosis monitored by externalized phosphatidylserine and the alteration of mitochondrial membrane potential (Fig. 3). Although the baseline system xC activity of HeLa S3 cells is proposed to be inhibited by L-Glu (10 mM) applied in this study (Fig. 5D), L-Glu (10 mM) alone did not reduce GSH level (Fig. 4B). In the absence of MeHg, the GSH level can probably be maintained in the normal range even under suppression of baseline system xC activity through the supply of intracellular cysteine via the other pathways.

System xC is known to be upregulated by oxidative stress (21, 33, 34). The molecular nature of system xC was identified as the heterodimeric protein composed of a catalytic unit xCT and an accessory unit 4F2hc (14, 35). We showed that mRNA of xCT was present in untreated HeLa cells was consistent with that of system xCT in its inhibitor sensitivity (Fig. 5). xCT mRNA was upregulated by MeHg and MeHg plus L-Glu. Cystine uptake was increased slightly by L-Glu and MeHg and largely augmented by the treatment with MeHg plus L-Glu. Although there is some discrepancy in the profiles of increment of mRNA and cystine uptake, the upregulation of xCT activity confirmed oxidative stress behind the phenomena observed in this study.

In the conditions used in the present study, the concentration of MeHg (8 μM) was determined so that MeHg alone exhibited minimal effects on HeLa cell viability, whereas its toxic effect was highly enhanced by 10 mM L-Glu. Thus, the MeHg (8 μM) alone did not show large effects on apoptosis, ROS level, and GSH level. However, MeHg (8 μM) alone still slightly increased apoptosis and ROS level and decreased GSH level at 12 h, confirming the oxidative stress induced by MeHg itself. This is consistent with the increase in xCT expression induced by MeHg alone (Fig. 5). L-Glu (10 mM) alone did not show significant effect on most of the parameters examined in this study. L-Glu alone, however, tended to increase xCT mRNA level at 12 h, although it was not statistically significant and slightly but significantly increased cystine uptake by the cells (Fig. 5), seemingly reflecting oxidative stress imposed by 10 mM L-Glu which might not be detected by ROS measurement. Therefore, the enhancement of toxicity by co-treatment with MeHg and L-Glu observed in this study was highly synergistic. Although the molecular mechanisms of this synergistic nature are not understood at this moment, it is possible that some other additional cellular events driven by L-Glu and MeHg could also contribute to modulate the process, which needs to be elucidated in the future.

In this study, non-neuronal cells were mainly used to characterize synergistic toxicity of MeHg and L-Glu. Although it was partly confirmed in neural cells (neuroblastoma cell lines), our results may not be directly applicable to the toxicity in neurons in brain tissues. Nevertheless, what we found seems implicated in MeHg toxicity at least partly in the central nervous system. One of the unsolved mysteries of MeHg neurotoxicity is the locality of the lesion. Discrete anatomical areas of the brain, such as visual cortex, other sensory cortex, and the granule layers of the cerebellum, are reported to be highly liable to the damage by MeHg (7). These brain areas are proposed to be highly active in processing sensory signals and other neural signals, so that it is possible that the neurons in these areas could be more exposed to the excitatory neurotransmitter L-Glu compared to the other parts of the brain. Whether the enhancement of MeHg toxicity observed in this study is involved in the mechanisms of locality of MeHg lesion would be an interesting issue to be examined in the future.

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