Glutamate-mediated effects of caffeine and interferon-γ on mercury-induced toxicity

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Abstract. The molecular mechanisms mediating mercury-induced neurotoxicity are not yet completely understood. Thus, the aim of this study was to investigate whether the severity of MeHg- and $HgCl_2$-mediated cytotoxicity to SH-SY5Y human dopaminergic neurons can be attenuated by regulating glutamate-mediated signal-transmission through caffeine and interferon-γ (IFN-γ). The SH-SY5Y cells were exposed to 1, 2 and 5 μM of either $MeHgCl_2$ or $HgCl_2$ in the presence or absence of L-glutamine. To examine the effect of adenosine receptor antagonist, the cells were treated with 10 and 20 μM caffeine. The total mitochondrial metabolic activity and oxidative stress intensity coefficient were determined in the 1 ng/ml IFN-γ- and glutamate-stimulated SH-SY5Y cells. Following exposure to mercury, the concentration-dependent decrease in mitochondrial metabolic activity inversely correlated with oxidative stress intensity. MeHg was more toxic than $HgCl_2$. Mercury-induced neuronal death was dependent on glutamate-mediated excitotoxicity. Caffeine reduced the mercury-induced oxidative stress in glutamine-containing medium. IFN-γ treatment decreased cell viability and increased oxidative stress in glutamine-free medium, despite caffeine supplementation. Although caffeine exerted a protective effect against MeHg-induced toxicity with glutamate transmission, under co-stimulation with glutamine and IFN-γ, caffeine decreased the MeHg-induced average oxidative stress only by half. Thereby, our data indicate that the IFN-γ stimulation of mercury-exposed dopaminergic neurons in neuroinflammatory diseases may diminish the neuroprotective effects of caffeine.

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Introduction

Mercury exposure is linked to a shift in the redox status toward oxidative stress. It may enhance lipid peroxidation in all tissues and may have deleterious effects on an organism (1). As MeHg easily crosses the blood-brain barrier, it is highly neurotoxic in exposed human populations (2). Therefore, its cytotoxic effect on neurons is stronger when compared to inorganic $HgCl_2$, even at low levels (3). Eventually, MeHg administration reduces non-enzymatic and enzymatic antioxidants (6).

Mercury has been shown to affect several aspects of glutamatergic signaling (4). In this context, MeHg markedly increases the glutamate concentration at the synaptic cleft by enhancing spontaneous glutamate release from neurons (5). Eventual excitotoxic activity of glutamate resulting from MeHg exposure contributes to neuronal injury. N-methyl-D-aspartate (NMDA) receptor-binding memantine attenuates MeHg-induced neurotoxicity (6). It has also been shown that the $HgCl_2$-induced reduction of cell viability is substantially attenuated by the application of a non-competitive antagonist of NMDA receptors (7). Although mercury-induced neuronal degeneration is suggested to invoke glutamate-mediated excitotoxicity, the underlying mechanisms remain poorly understood.

Caffeine is the most widely consumed psychoactive substance and acts as an antagonist of adenosine A1 and A2A receptors at non-toxic doses (8). Although A1 receptors are located pre-synaptically on dopaminergic, glutamatergic and cholinergic inputs to neurons, Brown *et al* could not detect any evidence regarding the effect of caffeine on mercury-induced toxicity (9).

On the other hand, mercury-exposed rats have been shown to exhibit enhanced interferon-γ (IFN-γ) serum levels as compared to the controls (10). Furthermore, it is claimed that vascular endothelial growth factor and interleukin-6 (IL-6) are released from human mast cells via the stimulation of mercury and disrupt the blood-brain-barrier and permit brain inflammation (11). In neurodegenerative diseases, brain inflammation and the facilitated entrance of immune cells through the blood-brain barrier can potentially cause neuronal damage and cognitive dysfunction (12,13). Thus, the disruption of the blood-brain barrier allows the infiltration of immune

cells to the brain and enhances the responsiveness of neurons to IFN-γ (14). T-cell traffic across the blood-brain barrier considerably increases, thereby exposing neuronal cells to the potent effects of IFN-γ (15). Eventually, IFN-γ acts directly on neural cells (16,17), and causes neurodegenerative alterations in the central nervous system (CNS) (18). Nevertheless, the precise role of IFN-γ during neuro-inflammation remains unclear (19). Mizuno *et al* suggested that IFN-γ synergistically enhances glutamate neurotoxicity mediated by α-amino-3-hy droxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, but not NMDA receptors (20). By contrast, Lee *et al* previously indicated that IFN-γ-mediated neuroprotection is associated with an enhanced recovery of intracellular Ca^{2+} concentrations following exposure to glutamate (21). In this manner, conflicting results have been presented regarding the effect of IFN-γ on glutamate-induced signaling. Furthermore, there is less information available on the association between mercury-induced cytotoxicity and caffeine or IFN-γ during the presence or absence of glutamine.

Thus, the aim of the present study was primarily to investigate whether mercury-induced neuronal damage is associated with glutamatergic excitotoxicity, and secondly, to determine whether glutamate signal transmission participates in the alteration of mercury-induced neurotoxicity through caffeine and IFN-γ.

Materials and methods

Cell culture. The human neuroblastoma cell line, SH-SY5Y, was cultured in EMEM:F12 (1:1) (Biochrom GmbH, Berlin, Germany) supplemented with 15% fetal bovine serum (FBS; Biochrom GmbH) at 37˚C, 5% CO_2. The cells were divided into 2 groups and cultured in either 292 mg/l L-glutamine containing or L-glutamine free-medium. All the experiments were run in both cell groups. The solutions of 1, 2 and 5 μM $MeHgCl_2$ and $HgCl_2$ (Merck KGaA, Darmstadt, Germany), 10 and 20 μM caffeine (Sigma-Aldrich, St. Louis, MO, USA) were prepared in L-glutamine-supplemented or glutamine-free medium (Biochrom GmbH) and sterilized using a 0.2 μm syringe filter (Fuxing Pharmaceutical Co., Ltd., Shanghai, China). Experiments were repeated under either 1 ng/ml human IFN-γ (hIFN-γ)-containing or hIFN-γ-free conditions.

Production of hIFN-γ. Active hIFN-γ was produced by using a bacterial protein expression system. The pET28a-based expression plasmid was constructed using the SLICE cloning procedure, as previously described (22). Briefly, codon optimized synthetic gene that encodes hIFN-g mature peptide (Uniprot accession P01579, amino acids between 24 and 161) was purchased from Macrogen, Inc. (Seoul, Korea). *Escherichia coli* BL21 cells were used as the expression host (Novagen Inc., Madison, WI, USA). pET expression system and expression host bacterium *E. coli* BL21 cells were obtained from Novagen Inc. (23). The cells were grown in 100 ml of terriffic broth until a turbidity of 0.5 absorbance was reached at OD600. Subsequently, culture was induced by using 1 mM IPTG (24). Following overnight expression, the cells were harvested and lysed using BPER reagent (Thermo Fisher Scientific, Waltham, MA, USA). IFN-γ from cleared lysate was purified using immobilized nickel affinity chromatography (GE Healthcare, Piscataway, NJ, USA). Imidazole removal and a polishing step were performed using sephadex G25 (GE Healthcare) gel filtration chromatography, as previously described (25).

Experimental design. The SH-SY5Y human neuroblastoma cells (10^4 cells/well) were seeded in 96-well plates. Twenty four hours after seeding (one cell cycle), the cells were exposed to various concentrations of $HgCl_2$ and $MeHgCl_2$ in medium with or without 292 mg/l L-glutamine for either 24 or 48 h. All the assays were performed in triplicates in 3 sets of experiments.

In this study, in order to clarify the mechanisms responsible for mercury-induced neuronal toxicity, we used two different substances in addition to the various concentrations of mercury compounds in SH-SY5Y cell cultures, caffeine and IFN-γ. The concentrations of mercury compounds and caffeine that were used in the experiments, were selected by the evaluation of possible exposure doses (26-30). The exposure duration was determined as one and two cell cycles. For further experiments, 10^4 cells were seeded in 96-well plates in medium with or without 292 mg/l L-glutamine; each set was individually pre-incubated for 30 min with 10 or 20 μM caffeine and after this period, 1, 2 or 5 μM of either $MeHgCl_2$ or $HgCl_2$ were added and the cells were incubated for 24 and 48 h in FBS-containing medium. Each set of experiments was repeated with cells pre-incubated with hIFN-γ. In all samples 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described below. The cells were also counted using trypan blue dye (Sigma-Aldrich) for each time point and for each concentration in each assay condition.

Mitochondrial metabolic activity. Mitochondrial metabolic activity was assessed by MTT assay according to a modified method of Mosmann (31). Briefly, the cells were exposed to the compounds and MTT dye (Serva, Heidelberg, Germany) (0.5 mg/ml in phosphate-buffered saline; Merck KGaA) was added to each well 4 h after the completion of the incubation period. Thereafter, the produced formazan crystals were solubilized by the addition of 10% SDS (Merck KGaA) in 1 N HCl solution (Merck KGaA). The resultant absorbance was measured spectrophotometrically (VersaMax ELISA Microplate Reader; Molecular Devices, Sunnyvale, CA, USA) at 550 nm with the reference wavelength of 690 nm.

Total nitrite and nitrate (Merck KGaA) (NO_3+NO_2; NOx) levels were measured using the Griess method, as previously described (32). The oxidative stress intensity coefficient (Q) was calculated by dividing the NOx produced per cell (cell count; Cc) to the total mitochondrial metabolic activity per cell (alteration in cell viability - MTT/Cc); Q = [(NOx/Cc)/MTT/Cc] (33).

Statistical analysis. The significance of the differences between the control and compound-treated cell groups were analyzed by a Student's t-test and a value of $P<0.05$ was considered to indicate a statistically significant difference. The calculations were performed using the statistical package SPSS, version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

The effects of L-glutamine in caffeine-supplemented medium on the total mitochondrial metabolic activity and oxidative stress in mercury-exposed SH-SY5Y cells are shown in Tables I-IV.

Table I. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the first 24-h incubation period with or without caffeine in glutamine-free medium.

	Without caffeine		10 μM caffeine		20 μM caffeine	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.4±0.16	0.18±0.13	114.7±1.33	0.17±0.03	100.3±0.15	0.16±0.01
MeHg 1 μM	91.9±0.18[a]	0.23±0.07	98.5±0.58	0.23±0.04	92.1±0.54	0.19±0.02
MeHg 2 μM	94.4±0.51	0.24±0.02	94.1±0.42	0.24±0.03	94.8±0.92	0.18±0.04
MeHg 5 μM	56.0±1.02[a]	0.35±0.04[a]	57.8±1.87[a]	0.26±0.01[a]	44.4±1.14[a]	0.43±0.22[a]
$HgCl_2$ 1 μM	104.3±0.47	0.18±0.05	99.1±0.21	0.18±0.08	114.2±2.11	0.14±0.02
$HgCl_2$ 2 μM	104.8±0.01[a]	0.23±0.06	114.1±13.4	0.16±0.04	95.6±0.54	0.15±0.00
$HgCl_2$ 5 μM	104.4±0.86	0.19±0.04	94.8±0.89	0.19±0.02	95.4±1.35	0.22±0.04

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability.

Table II. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the second 24-h incubation period with or without caffeine in glutamine-free medium.

	Without caffeine		10 μM caffeine		20 μM caffeine	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.8±0.22	0.23±0.16	111.1±0.77	0.15±0.05	111.2±0.55[a]	0.14±0.03
MeHg 1 μM	100.2±0.35	0.19±0.00	91.0±0.04[a]	0.32±0.07	107.2±0.76	0.14±0.03
MeHg 2 μM	58.2±0.26[a]	0.27±0.01	44.9±0.17[a]	0.40±0.09[a]	89.2±1.43	0.18±0.02
MeHg 5 μM	40.1±0.74[a]	0.39±0.06[a]	34.6±0.27[a]	0.43±0.00[a]	29.4±0.44[a]	0.50±0.08[a]
$HgCl_2$ 1 μM	85.0±0.31[a]	0.19±0.01	104.0±0.99	0.17±0.01	122.3±0.52[a]	0.12±0.04
$HgCl_2$ 2 μM	88.3±0.09[a]	0.18±0.02	107.6±0.88	0.18±0.07	127.1±1.94	0.12±0.01[a]
$HgCl_2$ 5 μM	89.2±0.91	0.19±0.03	116.1±0.23[a]	0.18±0.03	113.1±2.00	0.17±0.00

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability.

Only 5 μM MeHg led to a significantly higher oxidative stress intensity score and lower cell viability when compared with the controls in L-glutamine-free medium (P<0.05) at the first 24-h incubation period (Table I). However, at the end of the 48-h incubation period in glutamine-free medium, caffeine supplementation generated more marked oxidative stress and caused a significant decrease in cell viability, particularly in the 5 μM MeHg-exposed SH-SY5Y cells (Table II). Moreover, in glutamine-free medium, caffeine supplementation enhanced mercury-induced oxidative stress by approximately 10-28% at the end of 48-h incubation period (Tables I and II). Following the addition of L-glutamine to the medium, 5 μM MeHg increased oxidative stress by 88.6% and decreased the total mitochondrial metabolic activity/cell viability by 48.7% at the first 24-h in comparison to glutamine-free medium (P<0.05; Table III), whereas the oxidative stress intensity score increased by 118%, cell viability decreased by 26% in the 5 μM MeHg-exposed SH-SY5Y cells after 48-h of incubation in glutamine-containing medium when compared to the cells cultued in glutamine-free medium (Table IV). In the glutamine-containing medium, exposure to $HgCl_2$ resulted in a smaller increase in oxidative stress compared to exposure to MeHg. Cell viability was higher when the cells were exposed to $HgCl_2$ compared to MeHg (Tables III and IV). These results indicated that MeHg was more toxic than $HgCl_2$ to the SH-SY5Y cells.

Of note, the addition of L-glutamine to the incubation medium increased oxidative stress by 133 and 118% in the cells exposed to 2 and 5 μM MeHg, respectively, at the end of 48-h incubation period (Table IV). Furthermore, in the presence of L-glutamine, caffeine supplementation to 5 μM MeHg-containing medium decreased the oxidative stress scores by 69 and 68% for 10 and 20 μM caffeine, respectively. At the 48 h, the addition of 10 μM caffeine to the culture medium containing L-glutamine augmented cell viability by 93%, while 20 μM caffeine increased viability by 142%, in comparison to the SH-SY5Y cells exposed only to 5 μM MeHg. Moreover, incubation with 10 or 20 μM caffeine attenuated MeHg-induced toxicity in the presence of L-glutamine (P<0.05) (Table IV). Overall, caffeine ameliorated the cytotoxic effects of mercury at all concentrations. In these cases, following exposure to mercury, alterations in mitochondrial metabolic activity/cell viability inversely

Table III. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the first 24-h incubation period with or without caffeine in glutamine-containing medium.

	Without caffeine		10 μM caffeine		20 μM caffeine	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.5±0.33	0.19±0.07	102.40±1.12	0.21±0.07	101.1±0.33	0.18±0.03
MeHg 1 μM	82.2±0.74[a]	0.27±0.02[a]	103.20±0.57	0.24±0.02[a]	84.80±0.67[a]	0.24±0.05[a]
MeHg 2 μM	73.79±0.63[a]	0.36±0.03[a]	94.50±0.38	0.21±0.03	84.70±1.00	0.20±0.05
MeHg 5 μM	28.7±0.56[a]	0.66±0.02[a]	67.30±0.54[a]	0.28±0.01[a]	73.1±0.25[a]	0.26±0.04[a]
$HgCl_2$ 1 μM	91.32±0.17[a]	0.17±0.05[a]	102.5±1.27	0.16±0.04	103.5±2.59	0.22±0.04
$HgCl_2$ 2 μM	80.81±0.37[a]	0.36±0.07[a]	92.7±1.52	0.25±0.07	113.8±1.02	0.23±0.02
$HgCl_2$ 5 μM	73.65±0.02[a]	0.36±0.04[a]	105.9±0.55	0.24±0.04[a]	105.2±0.35	0.27±0.01[a]

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability.

Table IV. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the second 24-h incubation period with or without caffeine in glutamine-containing medium.

	Without caffeine		10 μM caffeine		20 μM caffeine	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	100.0±0.38	0.36±0.15	93.8±1.27	0.24±0.05	108.5±0.20[a]	0.15±0.02[a]
MeHg 1 μM	82.42±0.79[a]	0.33±0.02	111.7±1.26	0.24±0.04	104.5±0.87	0.22±0.01
MeHg 2 μM	53.09±0.04[a]	0.63±0.03[a]	79.5±0.23[a]	0.32±0.05	74.1±1.50[a]	0.18±0.02
MeHg 5 μM	29.60±1.74[a]	0.85±0.16[a]	57.0±1.57[a]	0.26±0.02[a]	71.7±0.02[a]	0.27±0.21[a]
$HgCl_2$ 1 μM	89.16±0.13[a]	0.25±0.11[a]	103.8±1.27	0.15±0.03[a]	117.1±0.09[a]	0.14±0.02[a]
$HgCl_2$ 2 μM	76.44±0.14	0.29±0.14	111.8±0.94	0.16±0.03[a]	107.0±0.18[a]	0.23±0.06
$HgCl_2$ 5 μM	61.12±0.40[a]	0.35±0.11	98.3±0.82	0.18±0.00	86.8±1.09	0.28±0.09

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability.

correlated with oxidative stress intensity scores. Furthermore, the increase in the NOx generation-related oxidative stress and the decrease in cell viability were also inversely proportional in a dose-dependent manner, particularly in MeHg-exposed cells in the presence of L-glutamine.

On the other hand, the addition of caffeine to the glutamine-free medium had no significant effect on $HgCl_2$-related oxidative stress and mitochondrial metabolic activity/cell viability. By contrast, caffeine supplementation to the glutamine-containing medium significantly attenuated MeHg- and $HgCl_2$-related toxicity at the matched doses and for all concentrations during the first and second 24-h incubation periods. These results were interpreted as the consumption of antioxidant capacity due to mercury-induced toxicity. Thus, the most striking toxicity was observed with 5 μM MeHg. It should be noted that a significant amount of extracellular glutathione is directly derived from glutamine. Culture in glutamine-free medium reduces cell proliferation and viability and abolishes glutathione excretion (34).

The effects of L-glutamine on IFN-γ- and caffeine-supplemented medium on the total mitochondrial metabolic activity and oxidative stress in mercury-exposed SH-SY5Y cells are shown in Tables V-VIII. Stimulation of the SH-SY5Y cells with IFN-γ in glutamine-free medium irregularly affected the caffeine-controlled mercury-induced toxicity when compared to cells exposed to mercury only. Moreover, 10 μM caffeine augmented 5 μM MeHg-induced oxidative stress by 125.6% in glutamine-free medium, when the medium was supplemented with IFN-γ at the end of the 48-h incubation period. When glutamine was added to the IFN-γ-containing medium, the SH-SY5Y cells were 42% less protected by 10 μM caffeine in comparison to only glutamine-containing medium. In glutamine-containing medium, 10 μM caffeine decreased 5 μM MeHg-induced oxidative stress by 58 and 69% at the first 24-h and second 24-h incubation periods, respectively. However, under co-stimulation with glutamine and IFN-γ, 10 μM caffeine reduced 5 μM MeHg-induced oxidative stress in the SH-SY5Y cells by 44 and 56% at the first 24-h and

Table V. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the first 24-h incubation period with or without caffeine in glutamine-free and IFN-γ-containing medium.

	Without caffeine		10 μM caffeine + IFN-γ		20 μM caffeine + IFN-γ	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.4±0.16	0.18±0.13	79.6±1.02[a]	0.23±0.05[a]	82.9±0.87	0.25±0.05
MeHg 1 μM	91.9±0.18[a]	0.23±0.07	98.6±1.68	0.27±0.03	109.2±0.74	0.19±0.09[a]
MeHg 2 μM	94.4±0.51	0.24±0.02[a]	102.6±0.41	0.19±0.00[a]	100.4±0.54	0.19±0.08[a]
MeHg 5 μM	56.0±1.02[a]	0.35±0.04[a]	65.5±0.51[a]	0.28±0.02	60.1±0.23[a]	0.33±0.07[a]
$HgCl_2$ 1 μM	104.3±0.47	0.18±0.05	96.2±1.22	0.19±0.01[a]	96.6±1.80	0.18±0.00[a]
$HgCl_2$ 2 μM	104.8±0.01[a]	0.23±0.06	97.3±0.93	0.19±0.04[a]	93.4±0.87	0.21±0.00[a]
$HgCl_2$ 5 μM	104.4±0.86	0.19±0.04	101.8±0.79	0.17±0.02[a]	91.9±0.35[a]	0.23±0.03[a]

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability; IFN-γ, interferon-γ.

Table VI. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the second 24-h incubation period with or without caffeine in glutamine-free and IFN-γ-containing medium.

	Without caffeine		10 μM caffeine + IFN-γ		20 μM caffeine + IFN-γ	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.8±0.22	0.23±0.16	92.6±1.51	0.30±0.03	121.0±1.36[a]	0.22±0.02[a]
MeHg 1 μM	100.2±0.35	0.19±0.00	79.7±0.28[a]	0.40±0.02[a]	88.4±0.45[a]	0.21±0.02[a]
MeHg 2 μM	58.2±0.26[a]	0.27±0.01	63.8±0.70[a]	0.33±0.02	83.2±0.52[a]	0.27±0.09
MeHg 5 μM	40.1±0.74[a]	0.39±0.06[a]	28.9±0.49[a]	0.97±0.11[a]	36.5±0.56[a]	0.46±0.09[a]
$HgCl_2$ 1 μM	85.0±0.31[a]	0.19±0.01	93.2±1.79	0.18±0.01[a]	93.4±0.78	0.19±0.03[a]
$HgCl_2$ 2 μM	88.3±0.09[a]	0.18±0.02	94.4±0.77	0.19±0.07[a]	95.4±0.89	0.19±0.00[a]
$HgCl_2$ 5 μM	89.2±0.91	0.19±0.03	94.4±0.93	0.21±0.12[a]	85.4±0.11[a]	0.25±0.02[a]

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability; IFN-γ, interferon-γ.

second 24-h incubation periods, respectively. This suggests that the IFN-γ-stimulated SH-SY5Y cells in glutamine-free medium almost remained unresponsive to mercury-induced toxicity despite caffeine supplementation (Tables V and VI). Eventually, at the second 24 h incubation period, the addition of IFN-γ and caffeine to glutamine-free medium significantly enhanced the toxicity of MeHg (p<0.05).

In the L-glutamine-containing medium, MeHg treatment decreased the average cell viability of IFN-γ-stimulated neuronal cells following caffeine supplementation in comparison to the controls (Tables VII and VIII). Following the stimulation of neuronal cells with IFN-γ, caffeine supplementation provided a partial improvement in MeHg toxicity in comparison to the unstimulated counterparts. When taking into account the mitochondrial metabolic activities and oxidative stress scores, IFN-γ and caffeine were more effective against $HgCl_2$-induced toxicity than MeHg. On the one hand, L-glutamine increased mercury-induced toxicity, but on the other hand, it was required for improving the effects of caffeine against mercury-induced toxicity in IFN-γ-stimulated SH-SY5Y cells.

Of note, the most effective concentration was 20 μM caffeine in recovering cell viability and oxidative stress intensity of the mercury-exposed cells, which were pre-treated with IFN-γ in L-glutamine-containing medium (P<0.05). The addition of IFN-γ to the glutamine-containing medium aggravated average cell viability of the 24- plus 48-h incubation periods by 15 and 22% in the 10 and 20 μM caffeine-stimulated cells, respectively. These findings were in accordance with the increase in the oxidative stress intensity score with the IFN-γ stimulation of MeHg-exposed cells. Similarly, when the mean values of the 24- and 48-h incubation periods were considered, the elevation of Q was 37 and 31% in the 10 and 20 μM caffeine supplemented medium, respectively. The IFN-γ stimulation of mercury-exposed SH-SY5Y cells in the glutamine-containing medium reduced the protective effects of caffeine.

Discussion

Glutamine is the primary precursor for the biosynthesis of the neurotransmitters glutamate and γ-aminobutyric acid. It

Table VII. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the first 24-h incubation period with or without caffeine in glutamine- and IFN-γ-containing medium.

	Without caffeine		10 μM caffeine + IFN-γ		20 μM caffeine + IFN-γ	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	99.5±0.33	0.19±0.07	92.1±1.91	0.17±0.01	107.4±1.44	0.23±0.06
MeHg 1 μM	82.2±0.74[a]	0.27±0.02[a]	82.60±1.21[a]	0.32±0.06	104.9±0.37	0.20±0.16
MeHg 2 μM	73.79±0.63[a]	0.36±0.03[a]	87.00±0.15[a]	0.20±0.07[a]	89.8±0.32	0.20±0.02[a]
MeHg 5 μM	28.7±0.56[a]	0.66±0.02[a]	59.50±0.72[a]	0.37±0.03[a]	54.3±1.03[a]	0.32±0.04[a]
$HgCl_2$ 1μM	91.32±0.17[a]	0.17±0.05[a]	99.1±0.81	0.20±0.01[a]	129.6±2.37	0.13±0.00[a]
$HgCl_2$ 2 μM	80.81±0.37[a]	0.36±0.07[a]	104.0±0.27	0.22±0.05[a]	99.5±0.48	0.23±0.02[a]
$HgCl_2$ 5 μM	73.65±0.02[a]	0.36±0.04[a]	103.4±0.85	0.18±0.02	95.3±0.17[a]	0.19±0.03[a]

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability; IFN-γ, interferon-γ.

Table VIII. Oxidative stress intensity coefficient and total mitochondrial metabolic activity/viability of MeHg- and $HgCl_2$-exposed SH-SY5Y human neuroblastoma cells at the end of the second 24-h incubation period with or without caffeine in glutamine- and IFN-γ-containing medium.

	Without caffeine		10 μM caffeine + IFN-γ		20 μM caffeine + IFN-γ	
	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)	MTT (%)	Q (μM/%)
Control	100.0±0.38	0.36±0.15	102.4±0.52	0.24±0.14	98.7±1.82	0.19±0.00[a]
MeHg 1 μM	82.42±0.79[a]	0.33±0.02	98.5±0.32	0.34±0.16	114.0±1.34	0.15±0.00[a]
MeHg 2 μM	53.09±0.04[a]	0.63±0.03[a]	107.1±0.67	0.27±0.07	108.3±1.07	0.16±0.05
MeHg 5 μM	29.60±1.74[a]	0.85±0.16[a]	46.7±0.50[a]	0.37±0.02[a]	58.8±0.73[a]	0.36±0.00[a]
$HgCl_2$ 1 μM	89.16±0.13[a]	0.25±0.11[a]	104.1±0.03[a]	0.17±0.05[a]	116.6±0.40	0.20±0.03[a]
$HgCl_2$ 2 μM	76.44±0.14	0.29±0.14	92.2±2.49	0.27±0.05	101.0±0.10	0.25±0.05[a]
$HgCl_2$ 5 μM	61.12±0.40[a]	0.35±0.11	91.0±1.20	0.39±0.02	85.2±1.28	0.23±0.08[a]

[a]P<0.05, mercury-exposed cells vs. matched caffeine + mercury-exposed cells. MeHg, methyl mecury; Q:[(NOx/Cc)/(MTT/Cc)], oxidative stress coefficient; MTT, mitochondrial metabolic activity/cell viability; IFN-γ, interferon-γ.

is proposed that *in vivo* glutamine is synthesized and released by astrocytes, and is then transported into the neuron for subsequent conversion to neurotransmitters (35). The uptake of glutamine by neurons is an integral step in the glutamate-glutamine cycle, and a major pathway for the replenishment of neuronal glutamate (36). Besides, glutamatergic neurons exhibit highly efficient transport systems to accumulate L-glutamine, one of the major precursors of glutamate (37). Glutamine re-appears in neurons before conversion back to glutamate by glutaminase (38,39). In this respect, without glutamine influx, SH-SY5Y cells cannot produce glutamate in glutamine-free medium (40). The neuroblastoma cell line, SH-SY5Y, expresses a novel form of phosphate activated glutaminase (PAG) which deamidates glutamine to glutamate and ammonia at high rates (41). Glutamate dyshomeostasis and oxidative stress have been identified as two critical mechanisms mediating MeHg-induced neurotoxicity. Glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) appear to be inhibited by MeHg exposure (6) (Fig. 1).

In neurons, mitochondrial metabolism of exogenous glutamine is mainly responsible for the net synthesis of glutamate, which is a neurotransmitter, but it is also necessary for the synthesis of glutathione, the main endogenous antioxidant (42). Thereby mitochondrial metabolic activity is very important with respect to glutamatergic neurotransmission and cell antioxidant capacity. The increased activity of GSH/glutamate-cysteine ligase (GCL) in the cytoplasm also leads to the concurrent elevation of GSH in the mitochondrial compartment (Fig. 1). Kaur *et al* demonstrated that treatment with 5 μM MeHg for 30 min led to a significant increase in ROS generation and reduction in GSH content (43). In a previous study, SH-SY5Y cells treated for 24 h with MeHg exhibited a significant reduction in glutathione peroxidase activity in the brain. There was a concomitant significant decrease in cell viability and an increase in apoptosis (44). In this context, MeHg may react readily with GSH, leading to the formation of a MeHg-SG adduct that is excreted into the extracellular space of SH-SY5Y human neuroblastoma cells (45). As an expected result, we found that

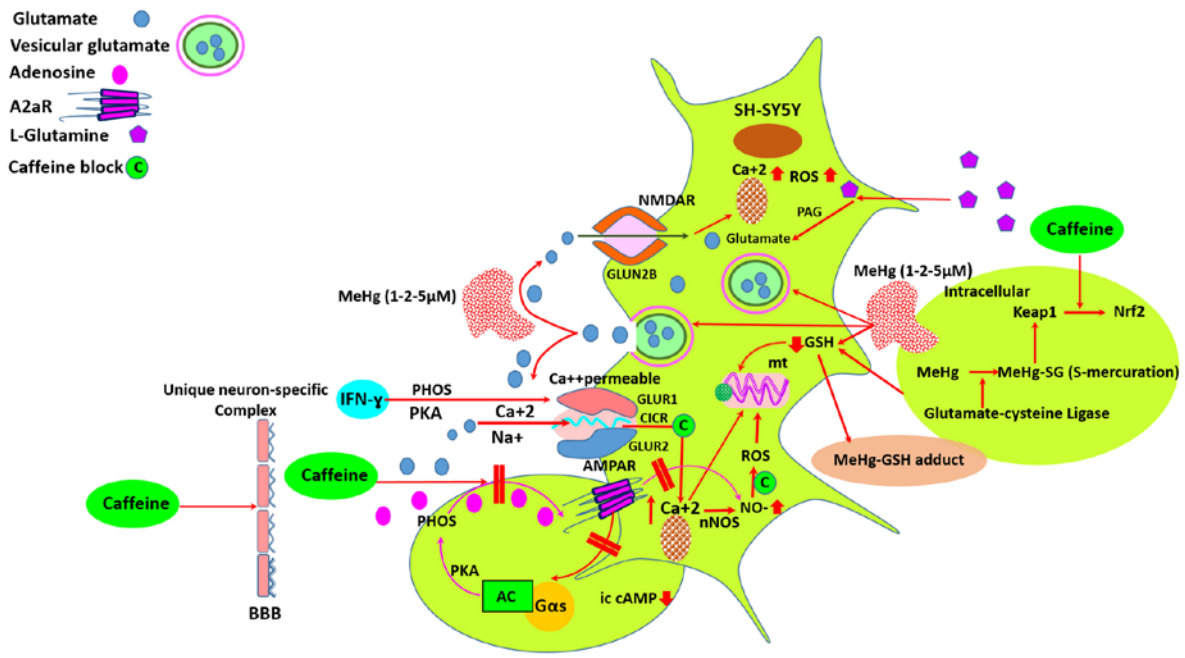

Figure 1. Mercury-induced neuronal death may occur via the glutamate-mediated excitotoxicity through NMDARs. Adenosine receptors blockade by caffeine equivalent doses of daily coffee consumption may reduce the vulnerability to mercury species-induced oxidative stress in L-glutamine contained medium. IFN-γ sensitizes the mercury-exposed SH-SY5Y dopaminergic neurons via AMPA receptor complex, and may diminish the neuroprotective effect of caffeine in the presence of L-glutamine. MeHg, methyl mercury; PAG, phosphate activated glutaminase; GSH, reduced glutathione; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (iontotropic glutamate receptor); IFN-γ, interferon-γ; mt, mitochondria; ROS, reactive oxygen species; nNOS, neuronal nitric oxide synthase; PKA, protein kinase A; A2aR, adenosine A2a receptor; PHOS, pphosphorylation of A2aR; AC, adenylyl cyclase; Gαs, stimulatory G-protein subunit; ic cAMP, intracellular second messenger cyclic adenosine monophosphate (cAMP); BBB, blood-brain barrier; CICR, calcium-induced calcium release; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Keap1, Kelch-like ECH-associated protein 1.

imercury-induced oxidative stress was not significantly affected in SH-SY5Y cells in glutamine-free medium, except, 5 μM MeHg exposure, whereas the toxic effects of mercury were significantly enhanced in L-glutamine-containing medium, particularly during the second 24-h incubation period.

Coffee consumption significantly reduced mercury-related toxicity. Of note, caffeine acting through adenosine receptors plays a prominent role in modulating glutamatergic input to various neurons (Fig. 1). Similarly, glutamate acts on both ionotropic and metabotropic G protein-coupled receptors. Therefore, intense glutamatergic neurotransmission is known to induce adenosine release (46). On the other hand, caffeine blocks calcium-induced calcium release (CICR) triggered by calcium influx through calcium permeable AMPA receptors (47). In our study, the addition of glutamine to the incubation medium increased the oxidative stress intensity by 89-118% in the 5 μM MeHg-exposed SH-SY5Y cells. Furthermore, caffeine supplementation to L-glutamine-containing incubation medium reversed the cytotoxicity of mercury compounds when compared with the caffeine-free controls. Caffeine-mediated effects may occur with at least two mechanisms: by directly blocking the glutamate activated channels or by reducing postsynaptic glutamate receptor density. In this respect, on the one hand caffeine decreases glutamatergic excitatory post-synaptic currents amplitude by direct postsynaptic block of glutamate-activated channels. On the other hand, caffeine directly blocks AMPA receptor-mediated calcium currents responsible for CICR (48). In daily life, caffeine concentration in blood reaches approximately 20-30 μM after ingestion of the equivalent of 2 cups of coffee (28). Thus, in our study, the maximum dose supplemented to the incubation medium was 20 μM. Indeed, the blood-brain barrier is readily permeable to caffeine, and thus the concentration in the brain is close to that in the blood (49). Caffeine inhibits glutamate receptors with an apparent IC_{50} value of approximately 10 mM. Therefore, ingested caffeine is unlikely to have any effect on ionotropic glutamate receptors. Instead, caffeine likely produces stimulatory effects in humans through its potent antagonism of the adenosine receptor (50). Caffeine-mediated glutamate receptor blockade may only occur under extreme conditions of toxicity (48).

Deletion of the A2A adenosine receptor reduces the vulnerability to MeHg, consistent with the neuroprotective effects of adenosine A2A receptor inactivation. Thus, MeHg toxicity can be reduced by adenosine A1 and A2A receptor inactivation, either via their genetic deletion or by treatment with their antagonist caffeine (51). In this study, in glutamine-free medium, caffeine did not block the toxicity of 5 μM MeHg. Thus, we observed a significant increase in the oxidative stress intensity score and a marked decrease in mitochondrial metabolic activity in the mercury-exposed SH-SY5Y cells. Substantially, MeHg disrupts glutamate metabolism and overexcites NMDA receptors of the neurons. At the same time, MeHg reduces non-enzymatic and enzymatic antioxidants, enhances neurocyte apoptosis, induces reactive oxygen species, and causes DNA peroxidative damage in the neurons (52). However, in our study, caffeine supplementation to glutamine-containing medium substantially ameliorated matched doses of MeHg- and $HgCl_2$-related toxicity. Caffeine-inhibited currents are activated by the direct application of glutamate to cortical neurons, confirming a post-synaptic site of action. This unexpected form of inhibition develops over tens of

milliseconds and is independent of NMDA receptors, consistent with non-NMDA receptor block (48). Furthermore, on human neuronal SH-SY5Y cells, caffeine shows concentration-dependent non-enzymatic antioxidant potential, decreases the basal levels of free radical generation, and reduces both superoxide dismutase and catalase activities (53). In addition, chronic coffee or caffeine ingestion reduces the lipid peroxidation in membranes of brain cells and increases the concentration of reduced-glutathione (54). We found that in glutamine-free medium, caffeine supplementation was insufficient to control 5 μM MeHg-induced oxidative stress. However, in glutamine-containing medium, caffeine inhibited MeHg-induced-oxidative stress by approximately 58 and 69% at the end of first and second incubation periods, respectively. These results confirmed that the antioxidant potential of caffeine was activated by glutamate, but was not mediated by NMDA receptor. In our study, we also demonstrated that equivalent doses of caffeine which were received during the daily coffee intake, substantially inhibited mercury-induced oxidative stress. However, NMDA receptor-mediated currents do not change in the presence of caffeine. Collectively, caffeine is a non-selective adenosine A1 and A2A receptor antagonist that attenuates dopaminergic neurotoxicity and neurodegeneration (55) (Fig. 1). It has been shown that pre-treatment with caffeine provides a partial neuro-protection against severe striatal degeneration in dopaminergic neurons and diminishes the extracellular glutamate in the brain (56).

Whether the effect of caffeine was mediated by a mechanism other than the NMDA receptor was examined by IFN-γ. IFN-γ is a pro-inflammatory cytokine that plays a pivotal role in the pathology of diseases in the CNS (20). Titze-de-Almeida *et al* demonstrated that IFN-γ sensitized SH-SY5Y cells to neurotoxin-induced injury, also causing an increase in ROS levels (57). Furthermore, IFN-γ directly induces neuronal dysfunction and enhances glutamate neurotoxicity mediated by AMPA receptors, but not NMDA receptors (20). Thus, in our study, IFN-γ in the pure SH-SY5Y cell culture worked synergistically with glutamate to promote neuronal excitotoxicity presumably through AMPA receptor complex in SH-SY5Y cells (Fig. 1). At the second 24-h incubation period, the addition of caffeine to IFN-γ-stimulated cells in glutamine-free medium significantly enhanced the toxicity of 5 μM MeHg. This result is in accordance with the findings of Titze-de-Almeida *et al* (57) and Vikman *et al* (58). Thus, Vikman *et al* indicated that when the neurons were treated with IFN-γ, neurophysiological alterations could be observed 48 h following exposure, when the frequency of AMPA receptor-mediated spontaneous excitatory post-synaptic currents are increased (58).

Caffeine supplementation could present a significant protective effect against MeHg toxicity with glutamate transmission. However, IFN-γ-stimulated neuronal cells were less protected by caffeine in L-glutamine-containing medium. Nevertheless, under the co-stimulation of SH-SY5Y cells with glutamine and IFN-γ, caffeine decreased MeHg-induced average oxidative stress by 50%. Glutamate seems to be an indispensable mediator of the effects of both mercury-induced toxicity and caffeine. Our results are in accordance with the findings of Bagga *et al*, with respect to glutamatergic neuronal activity and neurotransmission. Caffeine provides only partial neuroprotection against mercury-induced toxicity in IFN-γ-stimulated SH-SY5Y dopaminergic neurons (56).

In conclusion, these data suggest that mercury-induced neuronal death may occur through glutamate-mediated excitotoxicity. Adenosine receptor blockade by caffeine in equivalent doses of daily coffee consumption reduced the vulnerability to mercury-induced oxidative stress in glutamine-containing medium. The IFN-γ stimulation of SH-SY5Y dopaminergic neurons severely decreased cell viability and increased oxidative stress in glutamine-free medium despite caffeine supplementation. However, the addition of glutamine to the medium increased cell viability by 62% and reduced MeHg-related oxidative stress intensity by 62% in the presence of 10 μM caffeine. It can thus be concluded that the IFN-γ stimulation of mercury-exposed dopaminergic neurons in neuroinflammatory diseases may diminish the neuroprotective effects of caffeine.

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