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Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes

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Abstract

The neurotoxicity of high levels of methylmercury (MeHg) is well established both in humans and experimental animals. Astrocytes accumulate MeHg and play a prominent role in mediating MeHg toxicity in the central nervous system (CNS). Although the precise mechanisms of MeHg neurotoxicity are ill-defined, oxidative stress and altered mitochondrial and cell membrane permeability appear to be critical factors in its pathogenesis. The present study examined the effects of MeHg treatment on oxidative injury, mitochondrial inner membrane potential, glutamine uptake and expression of glutamine transporters in primary astrocyte cultures. MeHg caused a significant increase in F₂-isoprostanes (F₂-IsoPs), lipid peroxidation biomarkers of oxidative damage, in astrocyte cultures treated with 5 or 10 μ M MeHg for 1 or 6 hours. Consistent with this observation, MeHg induced a concentration-dependant reduction in the inner mitochondrial membrane potential ($\Delta\Psi_m$), as assessed by the potentiometric dye, tetramethylrhodamine ethyl ester (TMRE). Our results demonstrate that $\Delta\Psi_m$ is a very sensitive endpoint for MeHg toxicity, since significant reductions were observed after only 1 h exposure to concentrations of MeHg as low as 1 μ M. MeHg pretreatment (1, 5 and 10 μ M) for 30 min also inhibited the net uptake of glutamine (³H-glutamine) measured at 1 min and 5 min. Expression of the mRNA coding the glutamine transporters, SNAT3/SN1 and ASCT2, was inhibited only at the highest (10 μ M) MeHg concentration, suggesting that the reduction in glutamine uptake observed after 30 min treatment with lower concentrations of MeHg (1 and 5 μ M) was not due to inhibition of transcription. Taken together, these studies demonstrate that MeHg exposure is associated with increased mitochondrial membrane permeability, alterations in glutamine/glutamate cycling, increased ROS formation and consequent oxidative injury. Ultimately, MeHg initiates multiple additive or synergistic disruptive mechanisms that lead to cellular dysfunction and cell death.

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1. Introduction

Methylmercury (MeHg) is an organic form of mercury with well established neurotoxicity both in humans and experimental models [25,27]. Although the mechanisms of MeHg-induced neurotoxicity remain unclear, it is intriguing that a compound that readily reacts with sulfhydryl groups shows high organ specificity. MeHg can easily cross the blood-brain and placental barriers and cause central nervous system (CNS) damage in both the adult and developing brain [26,41]. While MeHg can directly cause damage to neurons, numerous studies have established a prominent role for astrocytes in mediating MeHg neurotoxicity [23,36]. The evidence includes observations that MeHg preferentially accumulates in astrocytes [5,18,34] and inhibits uptake systems for glutamate and cysteine transport, both of which will compromise glutathione (GSH) synthesis and redox status in astrocytes [2,16,30,61,62,63]. Furthermore, MeHg causes the activation of cytosolic phospholipase A₂ (cPLA₂), leading to arachidonic acid release and further inhibition of glutamate transporters and neuronal dysfunction [6,8].

Reactive oxygen species (ROS) generation has been linked to MeHg-induced toxicity both *in vivo* and *in vitro*. For example, cultured neurons [53] and glia [64] exposed to MeHg and brain synaptosomes prepared from animals injected with MeHg [1], demonstrate increased ROS production. In addition, an increase in ROS has been observed in mitochondria isolated from MeHg-injected rat brains, isolated rat brain mitochondria exposed to MeHg *in vitro* [54] and mitochondria from Hg- and glutamate-exposed astrocytes and neurons [14,32]. Evidence suggests that MeHg exposure causes production of ROS, depletion of ATP, excessive accumulation of calcium (Ca²⁺) and a decrease in mitochondrial membrane potential in mitochondria from the nervous [43] and immune [39,67] systems.

Excessive ROS production, leading to a decrease of mitochondrial membrane potential may also induce the oxidation of membrane polyunsaturated fatty acids, yielding a multitude of lipid peroxidation products. One such family of products is the F₂-isoprostanes (F₂-IsoPs), prostaglandin-like molecules produced by free radical-mediated peroxidation of arachidonic acid [52]. The measurement of F₂-IsoPs has emerged as the most accurate and reliable indicator of oxidative stress *in vivo* [57]. F₂-IsoPs levels are elevated in many tissues exposed to inflammation [45,46], excitotoxicity [47] and anticholinesterase toxicity [48], as well as in diseased regions of the brain in patients who died from advanced Alzheimer's disease [49]. However, biomarkers of free radical damage in the astrocyte model of MeHg neurotoxicity have not yet been evaluated.

Astrocytes play a primary neuromodulatory role in glutamate-glutamine homeostasis. Glutamine (Gln) is an important amino acid that plays a pivotal role in neuron-glia interactions, particularly in the turnover of the transmitter pool of glutamate (Glu) and γ -aminobutyric acid (GABA) [12,29,33], the principal CNS excitatory and inhibitory neurotransmitters respectively [24,28]. After being released during neurotransmission, glutamate is taken up largely by astrocytes. In the astrocytic compartment of the mammalian brain, glutamate is converted into glutamine via a highly active glutamine synthetase (GS) pathway and subsequently released into the intracellular space. Neighboring glutamatergic and GABAergic neurons use glial glutamine as a precursor for neurotransmitter synthesis as a part of the glutamate-glutamine cycle [11,59,60,68]. Studies in recent years have provided evidence that carrier-mediated Gln transport between astrocytes and neurons is a key factor in the glutamate/glutamine cycle. The molecular bases of Gln passage across the astrocytic membrane and neuronal plasma membranes have been investigated extensively over the last few years [12, 20,44]. Gln efflux from astrocytes appears to be mediated by sodium-coupled neutral amino acid transporter 3 (SNAT3, SN1), a system N amino acid transporter that is localized to perisynaptic astrocytes and specifically accepts only glutamine, histidine and asparagine [13, 19,21]. Gln uptake into neurons is mediated by sodium-dependent transporters of the system

A family, two of which, SNAT1 (GlnT, SAT1, ATA1, SA2) and SNAT2 (SAT2, ATA2), are thought to be capable of affecting Glu and/or GABA recycling, and, thereby, synaptic function. Another notable sodium-dependent, glutamine-accepting amino acid transporter in the CNS is ASCT2, which operates in an exchange mode. ASCT2 is more abundantly expressed in astrocytes than in neurons in culture [15,31]. A previous study from our laboratory has shown that exposure of astrocytes to acrylamide affects astrocytic glutamine uptake and expression of mRNA coding for Gln transporters [73] at concentrations comparable to those producing acute toxicity [7,9].

Given these earlier observations, the present study was carried out to examine the effects of MeHg treatment on oxidative injury (F_2 -IsoPs), mitochondrial inner membrane potential ($\Delta\Psi_m$) and both Gln uptake and expression of SNAT3, ASCT2 and SNAT1 mRNA in primary astrocyte cultures.

2. Materials and methods

2.1. Materials

L-[G- 3H]Glutamine (specific activity: 49.0 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Methylmercuric chloride (MeHgCl) was purchased from ICN Biomedicals (Costa Mesa, CA). Minimal essential medium (MEM) with Earle's salts, heat-inactivated horse serum, penicillin, streptomycin and tetramethyl rhodamine ethyl ester (TMRE) were purchased from Invitrogen (Carlsbad, CA).

2.2. Primary astrocytes culture

Astrocytic cultures from cerebral cortices of newborn (1-day-old) Sprague–Dawley rats were established as previously described [4]. Briefly, rat pups were decapitated, and the cerebral cortices were removed. After carefully removal of meninges, the cerebral cortices were digested with bacterial neutral protease (dispase, Invitrogen), and astrocytes were recovered by the repeated removal of dissociated cells from brain tissues. Twenty-four hours after the initial plating in 6- and 12-well plates, the media were changed to preserve the adhering astrocytes and to remove the neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air/5% CO₂ incubator for 3 – 4 weeks in minimal essential medium (MEM) with Earle's salts supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The media were changed twice per week. These monolayer, surface-adhering cultures were >95% positive for the astrocytic marker, glial fibrillary acidic protein (GFAP).

2.3. F_2 -isoprostanes Assay

Total F_2 -IsoPs were measured in primary astrocyte cultures using gas chromatography/mass spectrometry (GC/MS) with selective ion monitoring [52]. Briefly, cells were resuspended in 0.5 ml methanol containing 0.005% butylated hydroxytoluene, sonicated and then subjected to chemical saponification using 15% KOH to hydrolyze bound F_2 -IsoPs. The cell lysates were adjusted to pH 3, followed by the addition of 0.1 ng of 4H_2 -labeled 15- $F_{2\alpha}$ -IsoP internal standard. F_2 -IsoPs were then purified by C₁₈ and silica Sep-Pak extraction and by thin layer chromatography. They were then analyzed as pentafluorobenzyl ester, a trimethylsilyl ether derivative, via gas chromatography, negative ion chemical ionization mass spectrometry.

2.4. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was measured with the fluorescent dye TMRE [56]. Following MeHg treatments (1, 5 or 10 µM) for 1 or 6 hr, the culture medium was removed, and the cells were loaded with TMRE at a final concentration of 50 nM in HEPES buffer for 20 min at 37 °C in a 5% CO₂

incubator. At the end of treatment, cells were rinsed with PBS and examined with a Zeiss inverted fluorescent microscope (Zeiss Axiovert S100, Carl Zeiss MicroImaging Inc.) equipped with a cooled digital camera (Photometrics CoolSNAP, Roper Scientific Photometrics, Tucson, AZ). Images of various fields in each plate were captured at 10 $\times$ magnification with the digital camera. Fluorescent intensities were calculated in 8 randomly selected fields per experiment and were analyzed using the NIH software (Scion Incorporation, Frederick, MD). In each image field, the total number of pixels was quantified on a gray scale (0–255 counts), and the mean pixel value in each image field was obtained and expressed as mean $\pm$ S.E.M. The fluorescent intensities were expressed as percent fluorescence change over control. Each experiment was performed in duplicate plates and repeated three times using different astrocyte isolations.

2.5. Analysis of ^3H -glutamine uptake in astrocyte cultures

The ^3H -glutamine uptake assay was carried out as described previously [2]. Astrocytes were studied at 3 weeks when the monolayers were confluent. Cells in 6-well plates were washed three times with 2 ml of fresh sodium-HEPES buffer consisting of: 122 mM NaCl, 3.3 mM KCl, 0.4 mM MgSO_4 , 1.3 mM CaCl_2 , 1.2 mM KH_2PO_4 , 10 mM glucose, and 25 mM HEPES (N-2-hydroxy-ethylpiperazine N'-2-ethansulfonic acid) adjusted to pH 7.4 with 10 M NaOH. Cells were pretreated in Na-HEPES buffer only or with Na-HEPES buffer containing MeHg (1, 5, or 10 μM) for 30 min in a 37 $^\circ\text{C}$, in a 95% air/5% CO_2 incubator. Cells were then washed three times with 2 ml of Na-HEPES buffer, and, afterwards, rinsed with 1 ml of pre-warmed buffer containing 1 $\mu\text{Ci/ml}$ L-[G- ^3H]-glutamine in the presence of unlabelled glutamine. A final concentration of 50 μM was added to each well, and glutamine uptake was measured at 1 min and 5 min at room temperature. At each time point, the reactions were terminated by aspirating the buffer from the well, followed by 5 washes with 2 ml of ice-cold mannitol buffer [290 mM mannitol, 10 mM Tris-nitrate, 0.5 mM $\text{Ca}(\text{NO}_3)_2$, pH adjusted to 7.4 with KOH]. At the end of the experiment, cells were lysed in 2 ml of 1M NaOH. An aliquot of 25 μl was used for protein determination with the BCA protein assay (Pierce, Rockford, IL). An aliquot of 750 μl was used for radioactivity measurement. Uptake of glutamine was expressed as cpm/mg protein.

2.6. RT-PCR

Total RNA from cell cultures was extracted using Trizol (Invitrogen). RNA (2 μg) was transcribed using Superscript II (Invitrogen). The primers were obtained from the Laboratory of DNA Sequencing, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. Each cDNA (2 μl) was amplified by PCR using the primers for the rat amino acid transporters, SNAT3, SNAT1 and ASCT2. The expression was quantitatively related to the gene coding for the constitutive protein, GAPDH. The sequence of primers and the lengths of the products are outlined in Table 1. After 30 cycles of amplification (1 min at 94 $^\circ\text{C}$, 1 min at 59 $^\circ\text{C}$ for SNAT3, 55 $^\circ\text{C}$ for ASCT2 and SNAT1, and 1 min at 72 $^\circ\text{C}$ using Biometra thermocycler), the PCR products were recorded using the Nucleovision system (Nucleotech), and densitometric analysis was carried out using the GelExpert 4.0 program.

2.7. Statistical analysis

Measurements of ^3H -Glutamine uptake, F_2 -IsoPs levels and mitochondrial membrane potential were conducted in duplicate or triplicate wells/experiment, and the means from three to four experiments were used for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test with statistical significance set at $P < 0.05$. All analyses were carried out using Graph Pad Prism 4.02 for Windows (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1. Effects of MeHg on the F₂ – IsoPs formation in cultured astrocytes

We tested the ability of MeHg to induce oxidative stress in primary astrocytes by measuring levels of F₂-IsoPs, a lipid peroxidation biomarker of oxidative injury. Primary astrocytes exposed to 5 μ M or 10 μ M MeHg for 1 or 6 hours showed significant increases in F₂-isoPs levels ($p < 0.05$) (Fig. 1). The highest increases in F₂-IsoPs levels were detected after 6 hours with 5 μ M MeHg exposure. Concentrations $> 5 \mu$ M did not increase the effect of MeHg on F₂-IsoPs, suggesting a maximal effect at this concentration (5 μ M). The lowest MeHg concentration (1 μ M) did not cause a significant increase in markers of oxidative stress with F₂-IsoPs levels indistinguishable from controls.

3.2. Effects of MeHg on the $\Delta\Psi_m$ in cultured astrocytes

To determine whether mitochondrial dysfunction contributes to MeHg-induced neurotoxicity, we treated astrocytes with MeHg (1, 5 or 10 μ M) for 1 hour (Fig. 2A) and 6 hours (Fig. 2C) and measured the $\Delta\Psi_m$ by TMRE fluorescence. Treatments with MeHg for 1 hr (Fig. 2B) and 6 hr (Fig. 2D) resulted in significant dissipation of the $\Delta\Psi_m$, as demonstrated by decreased mitochondrial TMRE fluorescence. Quantification of TMRE fluorescence intensities revealed that all investigated MeHg concentrations induced significant dissipation of the $\Delta\Psi_m$.

3.3. Glutamine uptake inhibition by MeHg in cultured astrocytes

Compared with control uptake rates (100%), pretreatment of astrocytes for 30 min with MeHg inhibited the net uptake of glutamine at 1 min and 5 min in a concentration-dependent manner (Fig. 3). All MeHg treatments (1, 5, or 10 μ M) significantly ($p < 0.001$) inhibited astrocytic uptake at both time points. Furthermore, 10 μ M MeHg treatment induced significantly higher inhibition of glutamine uptake at 5 min compared to 1 or 5 μ M treatments ($p < 0.001$ and $p < 0.05$), respectively. No significant differences in glutamine uptake were seen between 1 and 5 min exposures when astrocytes were treated with 1, 5, or 10 μ M MeHg.

3.4. Influence of MeHg on expression of SNAT3, ASCT2, and SNAT1 mRNA

To explore molecular mechanisms associated with the effects of MeHg on glutamine uptake, we measured the astrocytic amino acid transporter mRNAs by RT-PCR. Figure 4 shows the effects of MeHg treatment on the mRNA expression of SNAT3, ASCT2, and SNAT1. The bars display the ratios of SNAT3, ASCT2 and SNAT1 to GAPDH, a constitutive marker. MeHg treatment at 10 μ M significantly ($p < 0.05$) reduced the mRNA expression of SNAT3 and ASCT2, but not of SNAT1 (Figures 4 A and B). The mRNA expression levels of all three amino acid transporters were not significantly different from controls ($p > 0.05$) when primary astrocytes cultures were exposed to 1 or 5 μ M MeHg.

4. Discussion

The present study established that MeHg exposure in astrocytes leads to lipid peroxidation and the induction of mitochondrial permeability transition. To our knowledge, this is the first study to investigate MeHg-induced neurotoxicity in astrocytes by employing biomarkers of oxidative damage (F₂-IsoPs) and fluorescent dye (TMRE) for measurement of $\Delta\Psi_m$. These damaging cellular changes initiated by MeHg will gradually lead to the dysfunction of astrocytes and contribute to their inability to maintain optimal control over the extracellular milieu, eventually leading to neuronal death.

In vivo and *in vitro* biochemical studies employing neuronal cultures and mixed neuronal/glial cultures as well as recent studies with primary astrocyte cultures have shown increased ROS formation with MeHg exposure [1,35,53,63,69,74]. Mitochondria are believed to be major

targets of MeHg-induced toxicity [43]. Specifically, highly enriched Hg concentrations were found in mitochondrial fractions with the lowest Hg concentration found in the cytosol of livers from Hg exposed animals [22]. Experiments with human hepatic cell lines also showed that almost half of the metal accumulates in the mitochondria where it causes ultrastructural alterations, as observed by transmission electron microscopy [40]. Most of the bioenergetic experiments with Hg report the uncoupling of oxidative phosphorylation [72], inhibition of ATP synthesis [10], impairment of the respiratory chain [58] and depletion of intracellular ATP and ADP [55]. A study using a selective probe for mitochondrial reactive oxygen intermediates as well as other probes demonstrated a significant MeHg-induced increase in intracellular superoxide anion, hydrogen peroxide and hydroxyl radicals, indicating that the mitochondrial electron transport chain is an early, primary site for ROS formation [2,65,66,74]. Additionally, MeHg exposure disrupts Ca^{2+} regulation in mitochondria derived from rat brains by decreasing Ca^{2+} uptake and inducing Ca^{2+} release [42]. Alterations in Ca^{2+} homeostasis can also lead to depolarization of the inner mitochondrial membrane and formation of ROS. Our results are consistent with studies showing that MeHg induces increases in ROS. Furthermore, our results extend this knowledge by showing that the lipid peroxidation biomarkers of oxidative injury, F_2 -IsoPs, are increased in MeHg exposed astrocytes.

Several methods exist to determine free radical-mediated damage to cells. While most of these function well *in vitro*, important limitations arise in living systems where extensive, highly active enzymatic pathways have evolved to metabolize many of the commonly measured products, such as 4-hydroxynonenal [50]. The measurement of F_2 -IsoPs is a method that has been extensively replicated as an efficient means of quantifying free radical damage in *in vivo* models associated with other neurodegenerative diseases, including Alzheimer's disease [49,51], inflammation [45,46], excitotoxicity [47] and anticholinesterase toxicity [48]. In our astrocyte model, levels of F_2 -isoPs were significantly elevated at 1 or 6 hours following 5 or 10 μM MeHg exposures (Figure 1).

Another consequence of increased oxidative stress is the induction of the mitochondrial permeability transition (MPT), a Ca^{2+} -dependent process characterized by the opening of the permeability transition pore (PTP) in the inner mitochondrial membrane. This causes increased permeability to protons, ions and other solutes ≤ 1500 Da [75], leading to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$). Loss of the $\Delta\Psi_m$ results in colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis and further generation of ROS. Our experiments demonstrated a concentration-dependent, deleterious effect of MeHg on mitochondrial $\Delta\Psi_m$ in cultured astrocytes. Our results also demonstrated that $\Delta\Psi_m$ is a very sensitive endpoint for MeHg toxicity, since significant reductions were observed after only 1 hour of exposure to MeHg at concentrations as low as 1 μM (Figure 2). These results agree with an earlier study by Limke and Atchison [43] that reported that mitochondria in primary cultures of rat cerebellar granule cells depolarize irreversibly after 50 min exposure to 0.5 μM MeHg. The mechanism by which MeHg induces the MPT in astrocytes is not completely understood, nor is the sequence of events that is associated with this effect. It is generally believed that increased $[\text{Ca}^{2+}]_i$ trigger ROS formation, and increased oxidative stress, in turn, is generally considered a major factor for MPT induction [17,38] and mitochondrial depolarization. However, the temporal sequence of events reported here with changes in membrane potential preceding oxidative stress suggest that changes in ionic gradients may be the earliest effects of MeHg. This is consistent with early reports from our laboratory showing that MeHg increases cellular permeability to ions such as Na^+ (and K^+), and that an increase in Na^+ permeability via Na^+/H^+ exchange, offsetting K^+ loss, is the primary mechanism in its inhibition of regulatory volume decrease in astrocytes [71].

Active, carrier-mediated efflux of Gln from astrocytes is a critical step in the Gln/Glu cycle, and, as such, a principal event in the neuromodulatory activity of these cells. Results from our study have shown that MeHg pretreatment for 30 min inhibited the net uptake of Gln at both 1 min and 5 min exposures. Under the present experimental conditions, uptake reflected active transport without directional distinction. Inhibition of Gln uptake in MeHg-treated astrocytes is therefore likely to reflect the decreased ability of cells to outwardly transport Gln. The two predominant Gln-transporting proteins of astrocytes are SNAT3(SN1) and ASCT2. Both are capable of mediating inward and outward transport of Gln, with the direction depending on the Gln and/or pH gradients [19]. The relative contribution of ASCT2- and SNAT3/SN1-mediated transport to the overall effect of MeHg remains to be established. Of note, inhibition of the expression of mRNAs coding for SNAT3/SN1 and ASCT2 was only observed at the highest MeHg concentration employed, indicating that, after 30 min treatment, inhibition of uptake was not due to inhibition of transcription. However, decreased expression of both transporters may become a cause for impaired Gln transport following prolonged exposure to MeHg, or, at later time points following a brief exposure. Experiments testing this hypothesis are currently being conducted.

Our results demonstrate that MeHg exposure is associated with changes in membrane permeability and glutamine/glutamate cycling increases in ROS formation and consequent lipid peroxidation. Furthermore, lipid peroxidation increases mitochondrial and cellular permeability alterations, involving GSH [37,70] and calcium depletion [70]. These outcomes work together to create a continuous cycle where acceleration of the mitochondrial chain induces oxidative stress, lipid peroxidation and depletion of antioxidant defenses, which, in turn, diminish membrane permeability and accelerate the respiratory chain, thus generating more ROS. Ultimately, the additive or synergistic mechanisms of cellular disruption caused by MeHg lead to cellular dysfunction and cell death.

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Abbreviations

$\Delta\Psi_m$	mitochondrial inner membrane potential
GABA	γ -aminobutyric acid
Gln	glutamine
Glu	glutamate
MeHg	Methylmercury
ROS	reactive oxygen species

Figure 1.

Effect of MeHg on F₂-IsoPs formation in cultured astrocytes. Rat primary astrocyte cultures were incubated at 37 °C in the absence or presence of MeHg (1, 5, and 10 μM) and F₂-IsoPs levels quantified at 1 and 6 hr, respectively. Data represent the mean ± S.E. from three independent experiments. * p<0.05 versus control by one-way ANOVA followed by Bonferroni multiple comparison tests.

Figure 2. Quantitation of TMRE fluorescent intensities. Cultured astrocytes exposed to MeHg at various concentrations (0, 1, 5, and 10 μ M) for 1 hr (A and B) and 6 hr (C and D) and the fluorescent

images quantified as described in Section 2. Values are expressed as mean $\pm$ S.E.M. of 24 random fields in each group. * $p < 0.05$, *** $p < 0.001$ versus control; $\Delta \Delta \Delta p < 0.001$ versus 10 μ M. note - (2A is image after 1h exposure; 2C is image after 6 h exposure).

Figure 3.

Effects of MeHg on glutamine uptake in astrocytes. Rat primary astrocyte cultures were incubated for 30 min at 37 °C in the absence or presence of MeHg (1, 5, and 10 μ M) and the net uptake of glutamine (3 H-glutamine) was quantified at 1 and 5 min, respectively. MeHg exposure induced a concentration-dependent decrease in glutamine uptake (***) $p < 0.001$ versus control; $\Delta p < 0.05$; $\Delta \Delta p < 0.001$ versus 10 μ M MeHg, $n = 6-10$; mean $\pm$ S.E.M.).

Figure 4.

Representative agarose gel electrophoresis of RT PCR products for the expression of SNAT3, ASCT2, SNAT1 mRNA (A). Expression of mRNA for SNAT3, ASCT2 and SNAT1 after treatment with MeHg (1 μM, 5 μM, 10 μM). Results are mean ± SD of three independent isolations. * < 0.05 vs. control (Mann -Whitney test) (B). Inset: GAPDH expression.

Table 1

Primer sequences used for RT-PCR analysis

Name	Primer (5'– 3')	Position	Size (bp)	GenBank number
Rat GAPDH	F tgaaggtcggagtcaacggatttg	80–104	998	BC013310
	R catgtaggccatgaggtccaccac	1062–1038		
SN1(SNAT3)	F aacatcggagccatgtccag	585–557	578	NM006841
	R aaggtgaggtagccgaagag	1159–1140		
ASCT2	F gccagtccacggccaagatc	1769–1750	687	BC080242
	R gcctggtcgtgttcgctata	1086–1102		
ATA1(SNAT1)	F cccattgtcactgctgagaa	1015–1034	681	NM181090
	R tccctgatatgtgggacaaa	1695–1676		