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Methylmercury-Induced Alterations in Astrocyte Function are Attenuated by Ebselen

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Abstract

Methylmercury (MeHg) preferentially accumulates in glia of the central nervous system (CNS), but its toxic mechanisms have yet to be fully recognized. In the present study, we tested the hypothesis that MeHg induces neurotoxicity via oxidative stress mechanisms, and that these effects are attenuated by the antioxidant, ebselen. Rat neonatal primary cortical astrocytes were pretreated with or without 10 μM ebselen for 2 hours followed by MeHg (0, 1, 5, and 10 μM) treatments. MeHg-induced changes in astrocytic [^{3}H]-glutamine uptake were assessed along with changes in mitochondrial membrane potential ($\Delta\Psi_m$), using the potentiometric dye tetramethylrhodamine ethyl ester (TMRE). Western blot analysis was used to detect MeHg-induced ERK (extracellular-signal related kinase) phosphorylation and caspase-3 activation. MeHg treatment significantly decreased ($p<0.05$) astrocytic [^{3}H]-glutamine uptake at all time points and concentrations. Ebselen fully reversed MeHg's (1 μM) effect on [^{3}H]-glutamine uptake at 1 min. At higher MeHg concentrations, ebselen partially reversed the MeHg-induced astrocytic inhibition of [^{3}H]-glutamine uptake [at 1 min (5 and 10 μM) ($p<0.05$); 5 min (1, 5 and 10 μM) ($p<0.05$)]. MeHg treatment (1 hour) significantly ($p<0.05$) dissipated the $\Delta\Psi_m$ in astrocytes as evidenced by a decrease in mitochondrial TMRE fluorescence. Ebselen fully reversed the effect of 1 μM MeHg treatment for 1 hour on astrocytic $\Delta\Psi_m$ and partially reversed the effect of 5 and 10 μM MeHg treatments for 1 hour on $\Delta\Psi_m$. In addition, ebselen inhibited MeHg-induced phosphorylation of ERK ($p<0.05$) and blocked MeHg-induced activation of caspase-3 ($p<0.05$ to 0.01). These results are consistent with the hypothesis that MeHg exerts its toxic effects via oxidative stress and that the phosphorylation of ERK and the dissipation of the astrocytic mitochondrial membrane potential are involved in MeHg toxicity. In addition, the protective effects elicited by ebselen reinforce the idea that organic selenocompounds represent promising strategies to counteract MeHg-induced neurotoxicity.

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Keywords

ebselen; methylmercury neurotoxicity; mitochondrial membrane potential; oxidative stress

Introduction

Methylmercury (MeHg) is an organic form of mercury (Hg) with toxic effects in multiple organs, and is one of the most poisonous environmental pollutants (Bakir et al., 1973; Takeuchi, 1989; Castoldi et al., 2008). It is a highly and selectively neurotoxic compound, leading to neurological and developmental deficits in the central nervous system (CNS), both in humans and experimental animals (Choi 1989; Clarkson et al., 2003; Pinheiro et al., 2008). MeHg preferentially accumulates in astrocytes and inhibits glutamate uptake in these cells. The toxic mechanism(s) of MeHg has yet to be fully understood (Aschner 2000).

Mitochondria, which are the main sites for the glutamate/GABA-glutamine cycle, represent a major target of MeHg (Allen et al., 2001). Earlier studies reported that cultured astrocytes ceased respiration at ~30 min after MeHg treatment, reflecting inhibition of the mitochondrial electron transport chain (Yee and Choi 1996; Allen et al., 2001; Shanker et al., 2004). MeHg-induced decrease of mitochondrial membrane potential has also been reported in neurons (Limke and Atchison 2002) and other cell types (InSug et al., 1997; Shenker et al., 1998). In the liver, MeHg has been shown to inhibit mitochondrial function, leading to K^+ influx and membrane depolarization (Sone et al., 1977).

Glutamine is an important precursor for the synthesis of the primary excitatory neurotransmitter glutamate and inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Boulland et al., 2002). Astrocytes-derived glutamine is taken up by neurons, where it is metabolized to glutamate, which, in turn, upon neuronal activity is released into the synaptic cleft and taken up by astrocytes via a Na^+-dependent mechanism. Subsequently, glutamate is converted to glutamine by a highly active glutamine synthetase (Sidoryk-Wegrzynowicz et al., 2009).

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is an organic selenium compound. Selenium is a structural component of several enzymes with physiological antioxidant properties, including glutathione (GSH) peroxidases (Flohé, et al., 1973), and it is known to possess anti-oxidant and anti-inflammatory properties (Cotgreave et al., 1989; Yang et al., 1999; Mugesh and Singh, 2000; Parnham and Sies, 2000). Of particular importance, the organoselenium compound, ebselen, has been demonstrated to be neuroprotective in preclinical studies (Saito et al., 1998; Davalos, 1999; Porciuncula et al., 2001; Satoh, et al., 2004; Centuriao, et al., 2005; Yamagata K et al., 2008). The antioxidant activity of organoselenides has been attributed to their GSH peroxidase-like activity (Muller et al., 1984; Wendel et al., 1984). More recently it has been demonstrated that ebselen is reduced by mammalian thioredoxin reductase (TrxR) forming ebselen selenol/selenolate (Zhao and Holmegren, 2002; De Freitas et al. 2010). Selenolate intermediates are potent nucleophiles and can readily react with electrophilic species, including reactive oxygen species (ROS) (Masumoto et al. 1996; Zhao and Holmegren 2002)

The present study was carried out to examine the effects of MeHg on glutamine metabolism and mitochondrial inner membrane potential ($\Delta\Psi_m$) in cultured astrocytes and to test the hypothesis that ebselen can effectively attenuate the toxicity of this metal. Additional studies addressed the efficacy of ebselen in attenuating MeHg-induced ERK phosphorylation and apoptosis via the activation of caspase-3.

2. Materials and methods

2.1. Materials

L-[G-^{3}H]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 tetramethylrhodamine ethyl ester (TMRE) were purchased from Invitrogen (Carlsbad, CA).

2.2. Primary astrocyte cultures

Methodologies for the isolation and culturing of cerebral cortical astrocytes derived from newborn (1-day-old) Sprague–Dawley rats were previously described (Yin at al 2007). In brief, rat pups were decapitated and the cerebral cortices removed. After carefully removing the meninges, the cerebral cortices were digested with bacterial neutral protease (Dispase, Invitrogen, Carlsbad, CA) and astrocytes recovered by repeated removal of dissociated cells from brain tissues. Twenty-four hours after the initial plating in BD Falcon 6- and 12-well plates, the medium was changed to preserve the adhering astrocytes and remove the neurons, microglia and oligodendrocytes. The cultures were maintained at 37°C in a 95% air / 5% CO_2 incubator for 3 weeks in MEM with Earle's salts supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed twice per week. These monolayer, surface-adhering cultures were >95% positive for the astrocytic marker, glial fibrillary acidic protein (GFAP).

2.3. Measurement of changes in mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was measured with the potentiometric dye tetramethylrhodamine ethyl ester (TMRE). TMRE accumulates in mitochondria as a function of the $\Delta\Psi_m$. At the end of treatments, the culture medium was removed (duplicate plates per experiment; repeated three times using different batches of astrocytes) and the cells were loaded for 20 min at 37°C in a 5% CO_2 incubator with TMRE at a final concentration of 50 nM in sodium-HEPES buffer. Cells were rinsed with phosphate buffered saline (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) controlled by computer software (Image Pro Laboratories, Stamford, CT). Images of various fields in each plate were captured at 10× magnification. Fluorescent intensities were obtained from 8 randomly selected fields per experiment and were analyzed with 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 was expressed as mean ± S.E.M. of the total number of mean pixel values in each group. The fluorescent intensities were expressed as percent fluorescence change over control.

2.4. Determination of the neuroprotective effects of ebselen on $\Delta\Psi_m$

Immediately after 2 hour pretreatment with or without ebselen (10 μM) in Na-HEPES buffer, MeHg was added for 1 hour to confluent astrocyte cultures (3 weeks post isolation) at 0, 1, 5, or 10 μM. Next, the cells were washed twice with 2 ml of Na-HEPES buffer and TMRE was loaded at a final concentration of 50 nM in Na-HEPES buffer for 20 min. Next astrocytes were washed with PBS and fluorescence was monitored as described above.

2.5. Western blot analysis

Astrocytes were treated with or without ebselen (10 μM) for 2 hours before exposure to MeHg (1, 5 or 10 μM) for various time periods. The cells were then lysed with lysis buffer

[Tris-HCl, pH 7.4, 20 mM, EDTA 2.5 mM, Triton X-100 1%, sodium deoxycholate 1%, SDS 0.1%, NaCl 100 mM, PMSF 1.0 mM, leupeptin 10 μg/ml, pepstatin 10 μg/ml] and collected for protein concentration determination by BCA assay (Pierce, Rockford, IL). An equal amount of protein (30 μg) was loaded and run on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (PerkinElmer Life Sciences, Boston, MA). The primary antibodies used were polyclonal anti-ERK1/2, monoclonal anti-phospho-ERK, polyclonal anti-caspase-3, and monoclonal anti-β-actin. The secondary antibodies were peroxidase conjugated (HRP) goat anti-rabbit IgG or goat anti-mouse IgG (Pierce, Rockford, IL). Supersignal West Pico (Pierce, Rockford, IL) was used for horseradish peroxidase (HRP) detection on a Hyperfilm ECL system (Nikon, Melville, NY). Stripping of the membrane was performed in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) as required. The levels of phosphorylated ERK (p–ERK) were expressed as arbitrary units of optical density, following the correction for content of total ERK (ERK1/2). Band intensities of caspase-3 were corrected for loading with β-actin. Densitometry measurement of band intensities was quantified and expressed as arbitrary units (AlphaEaseFC Imaging System software, Alpha Innotech, San Leandro, CA).

2.6. ^{3}H-Glutamine uptake in astrocytes

Astrocytes were studied 3 weeks post isolation, when the cell monolayer became fully 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. Immediately after 2 hour treatment with or without ebselen (10 μM) in sodium-HEPES buffer, astrocytes were treated in sodium-HEPES buffer only, or with sodium-HEPES buffer containing MeHg (1, 5, or 10 μM) for 30 min in a 37°C, 95% air / 5% CO_2 incubator. Cells were then washed three times with 2 ml of Na-HEPES buffer; thereafter, 1 ml of pre-warmed buffer containing 1 μCi/ml L-[G-^{3}H]glutamine 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 stopped 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 $Ca(NO_3)_2$, pH adjusted to 7.4 with KOH]. At the end of the experiments, cells were lysed in 2 ml of 1 M NaOH. An aliquot of 25 μl was used for protein determination with the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). An aliquot of 750 μl was used for radioactivity measurement by liquid scintillation and the values were expressed as cpm/mg protein (Tri-Carb 2900TR, Perkin Elmer Life Science).

2.7. Statistical analysis

^{3}H-glutamine uptake and release experiments were conducted in duplicate wells/experiment, and the mean from three to four independent experiments was used for statistical analysis. ^{3}H-Glutamine uptake and release were corrected for cellular protein levels and expressed as cpm/mg protein. TMRE staining was conducted in triplicate wells and the mean from three independent experiments was used for statistical analysis. Western blotting was performed three to four times. All data were expressed as percentage of control (100%) ± S.E.M. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple test with statistical significance set at $p<0.05$. When the overall significance resulted in the rejection of the null hypothesis ($p<0.05$), the source of the variance was determined with the Tukey-Kramer test (also known as the Tukey range test). All analyses were carried out with GraphPad software for Microsoft Windows (Graph Pad Software, San Diego, CA).

3. Results

3.1. Ebselen abolishes MeHg-induced astrocytic $\Delta\Psi_m$

Reactive oxygen species (ROS) generation has been linked to MeHg-induced neurotoxicity. To investigate the mechanisms by which MeHg induces $\Delta\Psi_m$, astrocytes were treated with various concentrations of MeHg for 1 hour, and $\Delta\Psi_m$ was measured by TMRE fluorescence. As shown in Fig. 1A, treatments with MeHg alone for 1 hour resulted in qualitative dissipation of the $\Delta\Psi_m$ in cultured astrocytes, as demonstrated by decrease in mitochondrial TMRE fluorescence. Quantification of TMRE fluorescent intensities (Fig. 1B) revealed that 1-hour treatment with MeHg at all tested concentrations (1, 5, and 10 μM) caused significant dissipation of $\Delta\Psi_m$. Ebselen alone (2 hour treatment) did not affect astrocytic $\Delta\Psi_m$ ($p>0.05$ compared with controls). Pre-treatment with ebselen (2 hours) followed by treatment with MeHg fully abolished the 1 μM MeHg-induced decrease in TMRE fluorescence ($p>0.05$ compared with control), and partially prevented the dissipation of the mitochondrial membrane potential in astrocytes treated with 5 or 10 μM MeHg.

3.2. Ebselen attenuates MeHg-induced ERK phosphorylation in astrocytes

The possible involvement of the mitogen-activated protein kinase (MAPK) subfamily in MeHg-induced neuronal injury was studied by measuring the phosphorylation levels of extracellular-signal regulated kinase (ERK). As shown in Fig. 3A, MeHg alone induced a robust increase in the levels of phosphorylated ERK in astrocytes as early at 15 min post exposure ($p<0.01$, compared with control) and the phosphorylation status remained elevated for at least 24 hours post MeHg treatment (Figs. 2A and 2B). Ebselen alone (2 hour treatment) did not increase the basal levels of phosphorylated ERK ($p>0.05$, compared with controls). Pretreatment with ebselen (2 hours) followed by MeHg exposure significantly ($p<0.05$ *vs*. MeHg alone) attenuated the effect of MeHg-induced ERK phosphorylation from 30 min to 24 hours of treatment (Figs. 2A and 2B).

3.43. Ebselen diminishes MeHg-induced caspase-3 cleavage in astrocytes

Next we investigated whether MeHg promotes caspase-3 cleavage in astrocytes. As shown in Fig. 3, MeHg treatment significantly promoted caspase-3 cleavage in astrocytes from 15 min to 6 hours. Ebselen alone (2 hour treatment) did not change caspase-3 cleavage values ($p>0.05$, compared with control). Combination treatments of ebselen (2 hour pretreatment) followed by MeHg exposure significantly prevented the MeHg alone-induced promoting effect on caspase-3 cleavage.

3.4. Ebselen reverses astrocytic MeHg-induced glutamine uptake inhibition

As shown in Fig. 4, MeHg (1, 5, and 10 μM) treatment alone for 30 min significantly inhibited in a concentration-dependent manner the astrocytic uptake of glutamine at 1 min ($p<0.01$ or $p<0.001$) and 5 min ($p<0.001$). Ebselen (10 μM) treatment alone for 2 hours did not affect astrocytic glutamine uptake ($p>0.05$ compared with control). Pre-treatment with ebselen displayed a significant protective effect against MeHg-induced glutamine uptake inhibition and this protection was observed at either 1 or 5 min.

4. Discussion

The present study demonstrates for the first time, that the antioxidant ebselen pretreatment stabilizes the mitochondrial membrane potential (Fig. 1), inhibits MeHg-induced ERK phosphorylation (Fig. 2) and attenuating the MeHg-induced promoting effect on caspase-3 cleavage (Fig. 3) and protects astrocytes from MeHg by restoring glutamine uptake (Fig 4),. Phosphorylation of ERK, caspase-3 cleavage and the collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) represent early events in MeHg-induced neurotoxicity

(Milatovic et al., 2007), which are linked to the demise in cellular homeostasis and ROS generation. Oxidative stress has been implicated in various neurodegenerative conditions as well as in metal-induced neurotoxicity (Bush, 2000). Studies in neuronal cultures, neuronal and glial co-cultures and recent studies in primary astrocytic cultures have all demonstrated increased ROS formation upon MeHg exposure (Ali et al., 1992;Gasso et al., 2001;Mundy and Freudenrich, 2000;Shanker et al., 2003;2005;Yin et al., 2007).

Ebselen is an organic selenium compound. Selenium is a structural component of several enzymes with physiologically antioxidant properties (Muller et al. 1984; Mugesh and Singh 2000). A number of selenium compounds possess chemical and biological antioxidant properties (Mugesh and Singh, 2000; Parnham and Sies, 2000; Imai et al., 2001; Nakamura et al., 2002; Herrera et al., 2003; Kalayci et al., 2005; Gabryel and Małecki, 2006; Johnsen-Soriano et al., 2007; Tripathi and Jena, 2008; Tak and Park, 2009). Ebselen was demonstrated to be neuroprotective in preclinical and clinical studies (Saito et al., 1998; Yamaguchi et al., 1998; Davalos, 1999) and in a variety of *in vitro* and *in vivo* animal models of neuropathological conditions, including ischemia (Dawson et al., 1995; Imai et al. 2003; Porciuncula et al., 2003), quinolinic acid- or glutamate-induced excitotoxicity (Porciuncula et al., 2001; Rossato et al., 2002a,b) and exposure to MeHg (Farina et al., 2003; Moretto et al., 2005; Funchal et al., 2006; Roos et al., 2009). The antioxidant activity of ebselen has been tentatively attributed to its GSH peroxidase-like activity (Muller et al., 1984; Wendel et al., 1984) and to its ability to serve as a substrate for mammalian thioredoxin reductase (TrxR), which metabolizes ebselen to its selenol/selenolate intermediate (Zhao and Holmegren, 2002; De Freitas et al. 2010). I*n vivo* treatment with ebselen and diphenyl diselenide can reduce MeHg neurotoxicity in rodents (Farina et al. 2003a,b; de Freitas et al. 2009). Accordingly, ebselen could have decreased MeHg toxicity in astrocytes as reported herein via its selenol by decreasing MeHg-induced oxidative stress and by a direct interaction of its selenol with MeHg, forming a non-toxic stable intermediate.

Our experiment also showed that MeHg, in a concentration-dependent manner, led to significant dissipation of $\Delta\Psi_m$ (Figure 1). These changes were fully or partially prevented by pretreatment with ebselen, attesting to the effectiveness of ebselen in reducing MeHg-induced ROS generation. Furthermore, as discussed above, the selenol intermediated of ebselen could interact directly with MeHg, thus decreasing its toxicity. Loss of the $\Delta\Psi_m$ leads to colloid osmotic swelling of the mitochondrial matrix (Gunter and Pfeiffer, 1990), redistribution of metabolites (Ca^{2+}, Mg^{2+}, Glutathione, NADPH) across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and the generation of ROS. These mitochondrial changes may initiate a cascade of events culminating in cell death (apoptosis or necrosis) (Berbardi et al., 1998;Kroemer and Reed, 2000). The $\Delta\Psi_m$ is a sensitive indicator for the energetic state of the mitochondria and the cell and can be used to assess the activity of the mitochondrial respiratory chain, electrogenic transport systems and the activation of the mitochondrial permeability transition (Ly *et al.*, 2003). Thus, evaluation of $\Delta\Psi_m$ depolarization is of critical importance for the assessment of cellular metabolism, viability and apoptosis.

Extracellular signal-regulated kinases (ERK1/2), one of the members of mitogen-activated protein kinase (MAPK), respond to several extracellular stimuli and are activated by MAPK/ERK kinase1/2 (MEK1/2) by phosphorylating threonine and tyrosine residues (Seger and Krebs 1995). It is known that oxidative stress activates MAPK cascades (Herrlich and Böhmer, 1999; Allen and Tresini 2000). ERK activation is generally considered a pro-survival pathway (Baines et al., 2002; de Bernardo et al., 2003), but increasing evidence suggests that phosphorylation of ERK also contributes to cell death (Chu et al., 2004; Zhuang and Schnellmann, 2006; Ren et al., 2009). The level of ERK phosphorylation or its

kinetics may play a role, as inhibiting basal ERK signaling has different effects than inhibiting toxin-induced ERK activation (Gomez-Santos et al., 2002). Furthermore, the time course of ERK activation is tightly correlated with mitochondrial ROS production and antioxidants inhibit ERK phosphorylation and rescue from neuronal injury (Chu et al., 2004; Kulich et al., 2007). ERK1/2 stimulation by ROS has been described in neurons (Samanta et al., 1998) and neuroprotection by MEK inhibition against oxidative stress in both neurons and in astrocytes (Satoh et al., 2000; Rosenberger et al., 2001). A rapid or transient activation of ERK promotes neuronal survival (Weng et al., 2007; Lin et al., 2008), while sustained or delayed ERK activation promotes cell death (Kulich and Chu, 2001; Gomez-Santos et al., 2002; Zhu et al., 2007). ERK can modulate mitochondrial functions (particularly those associated with cell death) and promote oxidative injuries (Alonso et al., 2004; Chu et al., 2004; Kulich et al., 2007; Dagda et al., 2008). In addition, activation of ERK is involved in the induction of apoptosis in cortical astrocytes (Blazquez et al., 2000; Oh et al., 2006). Our results showed that MeHg activates ERK phosphorylation in a time-dependent manner (Fig. 2A and 2B). While early ERK phosphorylation may be protective, sustained activation (for at least 24 hours) (Fig. 2B) is likely deleterious. Accordingly, the ability of ebselen to attenuate the time-dependent phosphorylation of ERK likely reflects its ability to protect astrocytes from the sequalae of sustained MeHg-induced ERK phosphorylation (Fig. 2A and 2B).

Caspase-3 plays a central role in mediating apoptosis, chromatin condensation and DNA fragmentation (Riedl and Shi, 2004). Therefore, caspase-3 is considered one of the major executioners of apoptosis and has classically been viewed as a terminal event in the process of programmed cell death. Accordingly, caspase-3 activation has been described in neuronal cells following specific types of central nervous system (CNS) insults, including traumatic brain injury and ischemic/excitotoxic damage (Beer et al., 2000; Nath et al., 2000; Brecht et al., 2001; Manabat et al., 2003). *In vitro* studies have also suggested that caspase-3 proteolytic activity plays a crucial role in excitotoxin-induced neuronal apoptosis (Allen et al., 1999; Tenneti and Lipton, 2000). In addition, expression of caspase-3 has been described in oligodendrocytes (Beer et al., 2000; Nottingham and Springer, 2003) and astrocytes following CNS damage (Beer et al., 2000; Benjelloun et al., 2003; Mouser et al., 2006). The present study also demonstrated that MeHg promotes caspase-3 cleavage and that pretreatment with ebselen partially prevents this effect (Fig. 3).

Glutamine is an energy substrate for most cells (Fox et al., 1996) and an important precursor for neurotransmitters glutamate, GABA, and in particular for GSH synthesis (Albrecht et al., 2007). Our present study indicates that MeHg inhibits the astrocytic uptake of glutamine in a concentration-dependent manner (Fig. 4), corroborating our previous reports (Allen et al. 2000; Aschner, et al. 1990; 1994; 2000; 2007; and Mutkus et al., 2005). Ebselen pretreatment (2 hours) effectively abolished the MeHg-induced reduction in glutamine uptake (Fig. 1), suggesting that it may restore the efficient cycling of glutamine between astrocytes and neurons, assuring optimal glutamine homeostasis. Mechanisms associated with decreased astrocytic glutamine uptake are related to inhibition of mRNA coding of the glutamine transporters, SNAT3/SN1 and ASCT2 (Yin et al., 2007; Sidoryk-Wegrzynowicz et al., 2009) and potentially direct inhibition of the transporter by ROS, as has been previously ascribed to glutamate uptake inhibition by MeHg (Shanker et al., 2004).

In summary, the present study demonstrates that MeHg exerts its toxic effects, at least in part, by inhibiting astrocytic glutamine uptake, collapsing the mitochondrial inner membrane potential, and triggering phosphorylation of ERK and activation of caspase-3. The selenium-containing compound, ebselen, can markedly attenuate these MeHg-induced effects. From a molecular point of view, both the thiol-peroxidase activity of ebselen and the chemical interaction of MeHg with selenol ebselen intermediate appear to be responsible for

the observed protective effects. These results indicate that organic selenocompounds represent promising strategies to counteract MeHg-induced toxicity, shedding light on new pharmacological modalities for treatment of MeHg poisonings.

Acknowledgments

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Abbreviations

$\Delta\Psi_m$	mitochondrial membrane potential
CNS	central nervous system
ERK	extracellular-signal related kinase
MeHg	methylmercury
ROS	reactive oxygen species
TMRE	tetramethylrhodamine ethyl ester

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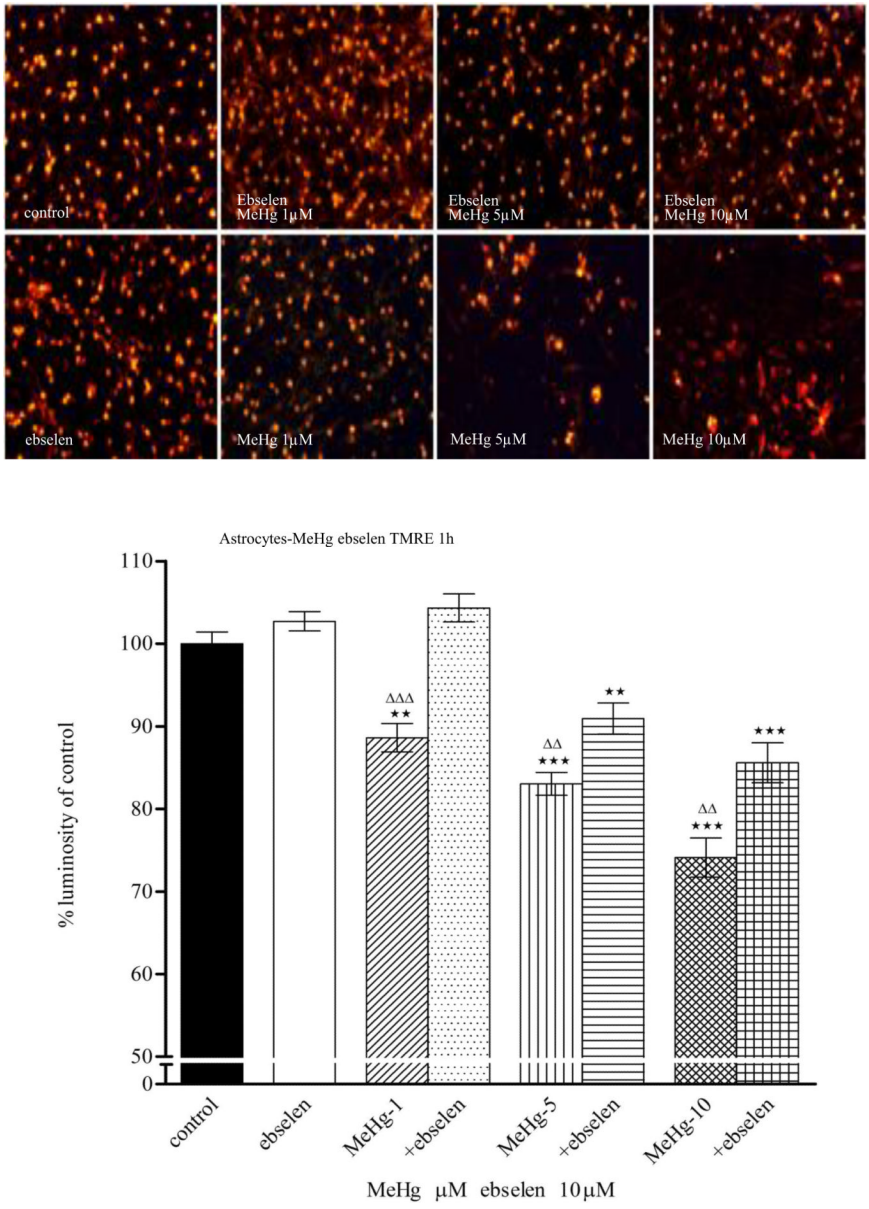

Figure 1.

Effects of MeHg/ebselen on mitochondrial ΔΨ. Cultured astrocytes were treated with or without ebselen (10 μM) for 2 hours and then were exposed to MeHg at various concentrations (0, 1, 5, or 10 μM) for 1 hour. (A) Fluorescent microscopy shows the mitochondrial ΔΨ after TMRE staining. (B) Quantitative analyses (see Materials and Methods Section) of TMRE fluorescence (ΔΨ). Values are expressed as mean ± SEM of 24 random fields in each group. Experiments were performed in three independently isolated sets of cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control; ΔΔΔ $p<0.001$ versus MeHg plus ebselen treatments in the paired groups by one-way ANOVA followed by Bonferroni multiple comparison tests.

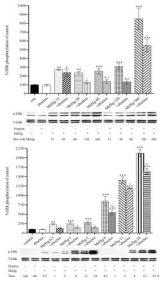

Figure 2.

Effects of MeHg/ebselen on ERK phosphorylation in cultured astrocytes as determined by immunoblotting (Figure 3A, 5 to 360 min; Figure 3B, 30 min to 24 hours). Prior to exposure to MeHg, astrocytes were pretreated with or without ebselen (10 μM) for 2 hours. Values are mean ± SEM of 4–6 independent experiments in each group. Statistical analysis was carried out by one-way ANOVA followed by Bonferroni multiple comparison tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control; Δ $p<0.05$; ΔΔ $p<0.01$ versus MeHg plus ebselen treatments in the paired groups.

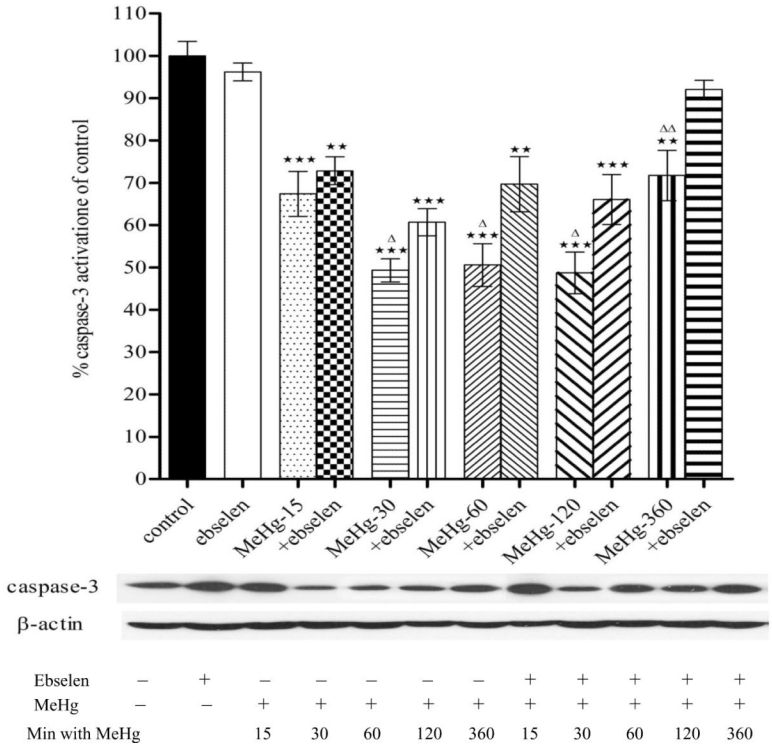

Figure 3.

Effects of MeHg/ebselen on activation of caspase-3 precursor in cultured astrocytes. Prior to exposure to MeHg, astrocytes were pretreated with or without ebselen (10 μM) for 2 hours. Values are mean ± SEM of 4–6 independent experiments in each group. Statistical analysis was carried out by one-way ANOVA followed by Bonferroni multiple comparison tests; ** $p<0.01$, *** $p<0.001$ versus control; Δ $p<0.05$; ΔΔ $p<0.01$ versus MeHg plus ebselen treatments in the paired groups.

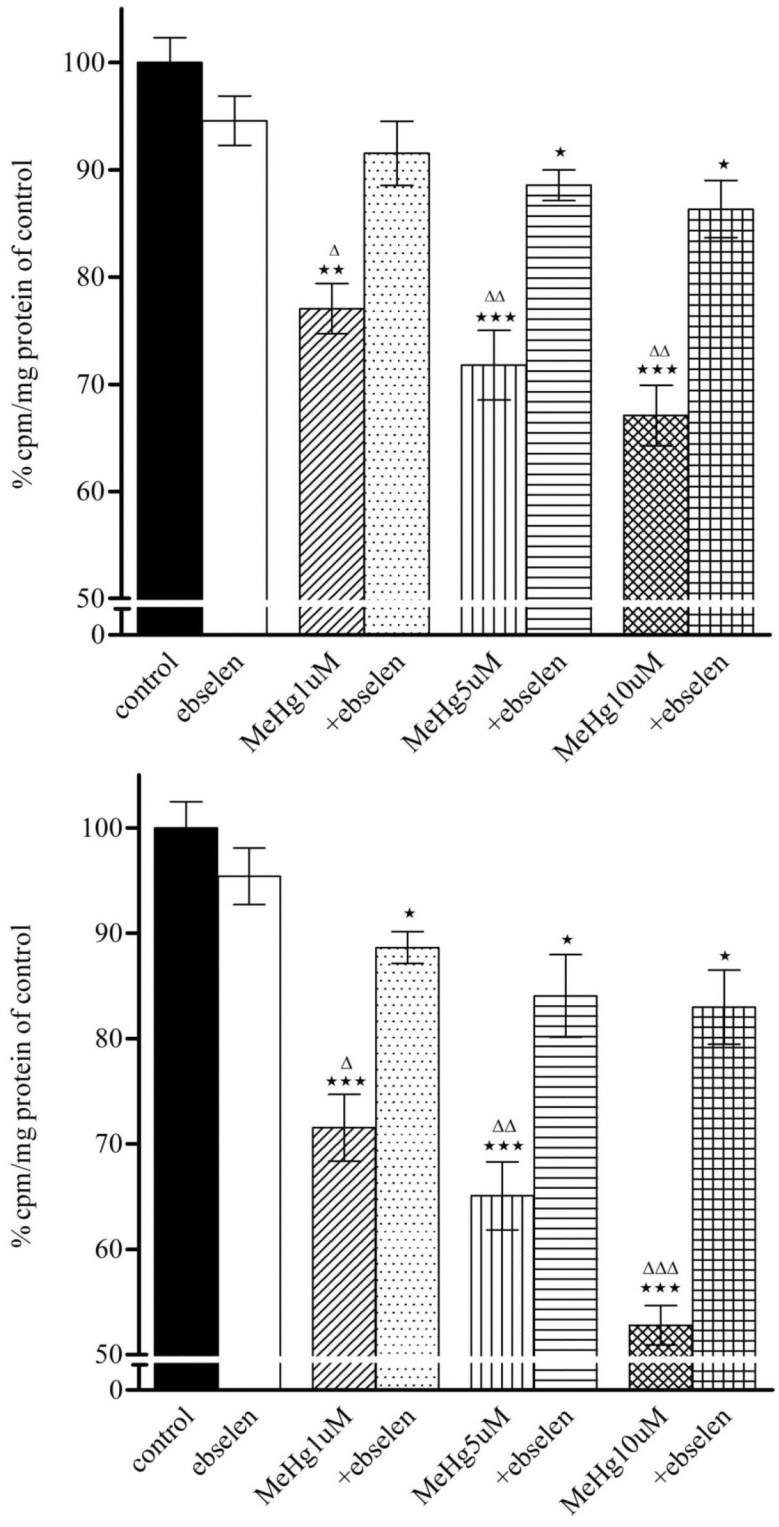

Figure 4.

Effects of MeHg/ebselen on glutamine uptake in astrocytes. Rat primary astrocytes cultures were pretreated with/without ebselen for 2 hours and then incubated for 30 min at 37°C in the absence or presence of MeHg (1, 5, or 10 μM), and the net uptake of glutamine (^{3}H-

glutamine) was quantified at 1 min (1A) and 5 min (1B), respectively. Values are expressed as, mean ± SEM (n=4–6). Experiments were performed in three independently isolated sets of cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control; Δ $p<0.05$; ΔΔ $p<0.01$, ΔΔΔ $p<0.001$ versus MeHg plus ebselen treatments in the paired groups by one-way ANOVA, followed by Bonferroni multiple comparison tests.