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Methylmercury-induced alterations in astrocyte functions 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 h 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 h) 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 h on astrocytic $\Delta\Psi_m$ and partially reversed the effect of 5 and 10 μM MeHg treatments for 1 h on $\Delta\Psi_m$. In addition, ebselen inhibited MeHg-induced phosphorylation of ERK ($p < 0.05$) and blocked MeHg-induced activation of caspase-3 ($p < 0.05$–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.

1. 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 et al., 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., 2001a,b). 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., 2001a,b; Shanker et al., 2004). MeHg-induced decrease of mitochondrial membrane potential has also been reported in neurons (Limke and Atchison, 2002) and other cell

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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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, 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; Porciúncula et al., 2001; Satoh et al., 2004; Centurião et al., 2005; Yamagata 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).

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 et 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 ± SEM 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 h pretreatment with or without ebselen (10 μM) in Na–HEPES buffer, MeHg was added for 1 h 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 h 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 and 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).