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Neuroprotection elicited by nerve growth factor and brain-derived neurotrophic factor released from astrocytes in response to methylmercury

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ABSTRACT

The protective roles of astrocytes in neurotoxicity induced by environmental chemicals, such as methylmercury (MeHg), are largely unknown. We found that conditioned medium of MeHg-treated astrocytes (MCM) attenuated neuronal cell death induced by MeHg, suggesting that astrocytes-released factors can protect neuronal cells. The increased expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) was observed in MeHg-treated astrocytes. NGF and BDNF were detected in culture media as homodimers, which are able to bind specific tyrosine kinase receptors, tropomyosin related kinase (Trk) A and TrkB, respectively. The TrkA antagonist and TrkB antagonist abolished the protective effects of MCM in neuronal cell death induced by MeHg. Taken together, astrocytes synthesize and release NGF and BDNF in response to MeHg to protect neurons from MeHg toxicity. This study is considered to show a novel defense mechanism against MeHg-induced neurotoxicity.

1. Introduction

Chemical forms of mercury are classified into metal mercury, inorganic mercury, and organic mercury. Methylmercury (MeHg) is an organic mercury compounds that is widely distributed in the environment. Nearly all MeHg in nature is converted from inorganic mercury via metabolism by microorganisms, and then MeHg is incorporated with microbes and concentrated in marine organisms through the food chain.

Once MeHg passes into the blood, it can easily pass through the blood–brain barrier as a cysteine conjugate mainly using the neutral amino acid transport system (Aschner and Clarkson, 1988, 1989). Thus, MeHg primarily targets the central nervous system (CNS), and induce abnormal behavior, depending on CNS disruption (Takeuchi, 1982). Furthermore, MeHg elicits sensory and auditory impairment in humans (Uchino et al., 1995), visual disturbance and tremors in monkeys (Charbonneau et al., 1974), and hind limb bending cross in rats (Klein et al., 1972). Some reports have suggested possible mechanisms of MeHg-induced neuronal injury, including disruption of microtubule, and apoptosis induced by reactive oxygen species (Dare et al., 2000). Interestingly, several small molecules and proteins are reported to attenuate MeHg-induced neurotoxicity. Vitamin E and selenium suppressed the decrease in body weight and neurologic symptoms, such as auditory response, observed in rodents following MeHg administration (Chang et al., 1978; Ganther, 1978; Beyrouty and Chan, 2006). Vitamin K and metallothioneine also attenuate MeHg-induced cytotoxicity in rat primary neurons or rat primary astrocytes, respectively (Prasad and Ramanujam, 1980; Zhang et al., 2009; Sakaue et al., 2011). In addition, we have reported that a neuroactive steroid, 17β-estradiol, attenuates neuronal cell death induced by MeHg in dentate gyrus of hippocampus (Yamazaki et al., 2013). Therefore, living animals are equipped with a diverse set of endogenous defense mechanisms against MeHg.

Nerve growth factor (NGF) was the first identified neurotrophin family protein. NGF promotes neurite outgrowth, synapse formation, and neuronal differentiation (Levi-Montalcini and Hamburger, 1951). At present, NGF, brain-derived neurotrophic factor (BDNF), neurotrohpin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) constitute the neurotrophin family in mammals. The receptors for these neurotrophins have also been identified. Tropomyosin-related kinase

Abbreviations: MeHg, methylmercury; CNS, central nervous system; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; Trk, tropomyosin related kinase; CM, conditioned medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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(Trk) A is a receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. Neurotrophin homodimers bind to each receptor to induce dimerization and autophosphorylation, followed by kinase signaling via a tyrosine kinase domain in the cytoplasmic region (Huang and Reichardt, 2003). There are some reports describing about the neuroprotective activity of neurotrophins. NGF is reported to inhibit cell death induced by rotenone in dopaminergic neurons (Jiang et al., 2006) and also suppressed neuronal damage elicited by oxygen and glucose deprivation in cortex neurons (Wu and Zhang, 1999). BDNF protected against cellular injury by 6-hydroxydopamine (6-OHDA) and N-methyl-4-phenylpyridinium ion (MPP^+) in dopaminergic neurons (Spina et al., 1992), and against glutamate excitotoxicity in hippocampal neurons (Almeida et al., 2005).

Recently, growing evidence indicates that astrocytes protect neuronal cells via neuroprotective factors released in response to neurotoxic stimuli. In case of dopamine toxicity, astrocytes are reported to release metallothionein to protect neurons (Miyazaki et al., 2011). Ischemia was shown to induce the synthesis and release of transforming growth factor-β1 and NT-3 in astrocytes, both of which inhibited cell death in primary neuronal cultures (Lin et al., 2006). Described above, neurotrophins are neuroprotective against several harmful stimuli, while BDNF reportedly exacerbates MeHg-induced neurotoxicity (Sakaue et al., 2009). In addition, it is also known that MeHg can modulate Trk signaling pathways (Parran et al., 2003). Therefore, effects of neurotrophins on MeHg toxicity remain controversial. Although neurotrophins are synthesized and released by neurons and astrocytes, astrocytes are regarded as the main source of neurotrophins in the brain because neurotrophins are expressed in astrocytes at all developmental stages and are constantly released from astrocytes (Schwartz and Nishiyama, 1994). MeHg dominantly accumulates in astrocytes in the central nervous system (Aschner et al., 1990), suggesting that astrocytes are the main target of MeHg in the CNS. Thus, the purpose of this study was to investigate the neuroprotective action of astrocytes exposed to MeHg, focusing on neurotrophins released from astrocytes.

2. Materials and methods

2.1. Materials

MeHg chloride (10 mM; Kanto Chemical, Tokyo, Japan) was dissolved in PBS with L-cysteine (10 mM) to form MeHg–cysteine complexes. The anti-BDNF antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The anti-NGF antibody was purchased from Gene Tex (Irvine, CA, USA). The anti-α-tubulin antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). GW-441756 was purchased from Focus Biomolecules (Plymouth Meeting, PA, USA). Cyclotraxin-B was synthesized by Eurofins Genomics (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries Limited (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma–Aldrich and were of reagent grade.

2.2. Cultures of rat cortex primary astrocytes

All procedures performed on animals were in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan and the Animal Care and Use Committee of Hiroshima University, Hiroshima, Japan.

Primary astrocyte cultures were prepared from the cerebral cortex of 1–2-day old male Wister rats (CLEA Japan, Tokyo, Japan), according to a previous protocol (Gatson and Singh, 2007). The cerebral hemispheres were excised, and the meninges were removed. The cerebral cortex was cut into small pieces and treated with 2.5% trypsin and 0.5% DNaseI for 20 min at 37 °C. After centrifugation (400 × *g*, 5 min), the pellets were gently suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and then centrifuged again. The pellets were resuspended in DMEM with 10% FBS and filtered using a Cell Strainer (70 μm, Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). The cells were plated at a density of 8×10^5 cells/well in 6-well plates coated with poly-L-lysine. The cultures were maintained in a humidified chamber with 5% CO_2/95% air at 37 °C. The medium was changed every two days after shaking the plates at a 250 rpm/min to remove other glial cells. The cells were cultured for 14–20 days with DMEM containing 10% FBS and for 3 days with FBS-free DMEM prior to experiments. The purity of the astrocyte-enriched cultures was confirmed by staining with antibodies against the astrocyte-specific marker, glial fibrillary acidic protein (GFAP). Over 95% of cultured astrocytes showed immunoreactivity for GFAP.

2.3. Cultures of SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were purchased from American Type Culture Collection (CRL-2266; Manassas, VA, USA). Cells were cultured and maintained according to our previous report (Ishihara et al., 2015). Cells were plated in a 96-well plate at a density of 8×10^4 cells/well and then cultured for 24 h. After incubating with FBS-free DMEM for 24 h, the cells were used for experiments.

2.4. Rat hippocampal organotypic slice cultures

Rat hippocampal slices were prepared from 9–10-day-old male Wister rats and were cultured as previously described (Ishihara et al., 2013). Briefly, hippocampi were dissected and then cut transversely into 300 μm slices. The slices on the Omnipore membranes (Millipore, Bedford, MA) were precultured for 4 days. After the culture media was changed to the serum-free medium, reagents were added to the culture.

2.5. Preparation of conditioned medium

Astrocytes were treated in the presence or absence of 3 or 10 μM MeHg for 24 h. The media were collected and centrifuged at 10,000 × *g* for 10 min. Supernatant was dialyzed to remove MeHg using Amicon Ultra (Millipore, Billerica, MA, USA). The resultant solutions were used as conditioned medium for 3 μM MeHg-treated (3MCM), 10 μM MeHg-treated (10MCM) and untreated (UCM) astrocytes.

2.6. Assay for cell viability

Cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. MTT solution (1/10 total volume; 5 mg/mL in PBS) was added to the culture medium and incubated for 2 h at 37 °C. After removing the culture medium, the formed formazan was dissolved with isopropanol containing 0.04 M HCl, followed by measurement of absorbance at a wavelength of 570 nm. The values of the untreated cells were considered 100%.

2.7. Measurement of PI uptake

Propidium iodide (PI) is a polar compound that only enters cells with damaged membranes and becomes brightly red fluorescent after binding nucleic acids. A concentration of 1 μM PI