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β-*N*-methylamino-L-alanine induces oxidative stress and glutamate release through action on system Xc^-

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ABSTRACT

β-*N*-methylamino-L-alanine (BMAA) is a non-protein amino acid implicated in the neurodegenerative disease amyotrophic lateral sclerosis/Parkinson–dementia complex (ALS/PDC) on Guam. BMAA has recently been discovered in the brains of Alzheimer's patients in Canada and is produced by various species of cyanobacteria around the world. These findings suggest the possibility that BMAA may be of concern not only for specific groups of Pacific Islanders, but for a much larger population. Previous studies have indicated that BMAA can act as an excitotoxin by acting on the NMDA receptor. We have shown that the mechanism of neurotoxicity is actually three-fold; it involves not only direct action on the NMDA receptor, but also activation of metabotropic glutamate receptor 5 (mGluR5) and induction of oxidative stress. We now explore the mechanism by which BMAA activates the mGluR5 receptor and induces oxidative stress. We found that BMAA inhibits the cystine/glutamate antiporter (system Xc^-) mediated cystine uptake, which in turn leads to glutathione depletion and increased oxidative stress. BMAA also appears to drive glutamate release via system Xc^- and this glutamate induces toxicity through activation of the mGluR5 receptor. Therefore, the oxidative stress and mGluR5 activation induced by BMAA are both mediated through action at system Xc^-. The multiple mechanisms of BMAA toxicity, particularly the depletion of glutathione and enhanced oxidative stress, may account for its ability to induce complex neurodegenerative diseases.

Introduction

In the 1959's it was observed that a substantial number of Chamorros, the native people of Guam, began developing a disease which showed combined symptoms of amyotrophic lateral sclerosis and Parkinson's dementia complex (ALS/PDC). The idea that β-*N*-methylamino-L-alanine (BMAA) may be involved in this disease began in the 1989's when it was found that BMAA was present in cycad seeds which were consumed by the Chamorros (Nunn et al., 1987) and that injection of BMAA into monkey brains induced a Parkinson-like disease (Spencer et al., 1987). Since its initial proposal, the BMAA hypothesis for the development of ALS/PDC on Guam has been controversial (Cox and Sacks, 2002; Papapetropoulos, 2007). It was challenged by findings that the levels of BMAA in cycad seeds are too low to cause damage to the brain or the spinal cord, particularly because the Chamorros thoroughly wash the cycad seeds, leading to very low levels of BMAA being consumed (Duncan et al., 1990). The BMAA hypothesis was largely abandoned until the last few years.

A number of recent studies have brought the BMAA hypothesis back into prominence. First, it was shown that BMAA is biomagnified. BMAA is produced by cyanobacteria that live on cycad plants; it accumulates in the cycad seeds, which are eaten by fruit bats, which are in turn eaten by the Chamorros (Cox et al., 2003). Second, it was shown that BMAA can become protein-associated. This property allows for BMAA to build up in tissue and provides a mechanism for slow release (Murch et al., 2004a). This slow release may provide a possible explanation for the delayed onset of ALS/PDC following the time of BMAA consumption (Ince and Codd, 2005). Third, cyanobacteria present throughout the world have been shown to produce BMAA (Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008; Johnson et al., 2008; Metcalf et al., 2008). Also, BMAA was found not only in brain samples of ALS/PDC patients from Guam, but also in the brains of Alzheimer's disease patients from Canada, but not in patients who died of other causes (Murch et al., 2004b). These results suggest that BMAA may be of concern not only for people on select Pacific islands, but for a much larger population. Fourth, BMAA at lower concentrations than previously believed are neurotoxic. The original studies in cortical cell culture found that very high BMAA concentrations (1–3 mM) were required to induce neuronal death (Ross et al., 1987; Weiss and Choi, 1988; Weiss et al., 1989). A more recent study found that BMAA concentrations as low as 30 μM can cause selective death of motor neurons (Rao et al., 2006), and we found that BMAA concentrations as low as 10 μM can enhance neuronal death

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induced by amyloid-β or 1-methyl-4-phenylpyridinium ion (MPP+) (Lobner et al., 2007).

Given the potential relevance of BMAA consumption to neurodegenerative diseases it is important to determine the mechanism of BMAA induced neuronal death. We have previously shown that BMAA induces neuronal death through 3 distinct mechanisms, activation of NMDA and mGluR5 receptors, and induction of oxidative stress (Lobner et al., 2007). Through electrophysiological recording it has been shown that BMAA directly acts on the NMDA receptor (Ross et al., 1987; Weiss and Choi, 1988; Brownson et al., 2002; Lobner et al., 2007). The current studies were designed to determine how BMAA activates mGluR5 receptors and induces oxidative stress. Evidence is presented that the cystine/glutamate antiporter (system Xc^-) plays an important role in these effects. System Xc^- involves the transport of cystine into the cell in exchange for glutamate being transported out of the cell. Given the functions of system Xc^- it seems likely that it plays an important role in neuronal survival and death. By releasing glutamate it can increase extracellular glutamate levels and potentially cause excitotoxicity. Through providing cystine uptake it can regulate cellular glutathione levels and in this way determine whether oxidative stress induced neuronal death will occur. We find that the effects of BMAA on neuronal death involve actions on system Xc^- to both inhibit cystine uptake and increase glutamate release.

Materials and methods

Materials

Timed pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, DE, USA). ^{35}S-cystine was from PerkinElmer Life and Analytical Sciences (Boston, MA). 5-(and -6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) was from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma (St. Louis, MO).

Cortical cell cultures

Mixed cortical cell cultures containing neuronal cells were prepared from fetal (15–16 day gestation) mice as previously described (Lobner, 2000). Dissociated cortical cells were plated on 24 well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Cultures were maintained in humidified 5% CO_2 incubators at 37 °C. Mice were handled in accordance with a protocol approved by our Institutional Animal Care Committee.

Induction of neuronal death

All experiments were performed on mixed cultures 13–15 days in vitro (DIV). Toxicity was induced by exposure to the toxic agents for 24 h in media as described for plating except without serum. All exposure media contained 26 mM $NaHCO_3$, as it has been shown previously that HCO_3^- is required for expression of NMDA receptor mediated BMAA toxicity (Weiss and Choi, 1988).

Assay of neuronal death (LDH release)

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 h after the beginning of the insult. Blank LDH levels were subtracted from insult LDH values and results normalized to 100% neuronal death caused by 500 mM NMDA. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (Koh and Choi, 1987; Lobner, 2000). Glial cell death (assessed by trypan blue staining) was not observed in any of the current studies. Therefore results are presented as percent neuronal death.

^{35}S-cystine uptake

Uptake of cystine was measure by exposure of cultures to ^{35}S-cystine (2 μCi/ml) for 20 min in the presence or absence of 3 mM BMAA and/or 1 mM S-4-carboxyphenyl glycine (CPG). Following the exposure to ^{35}S-cystine, the cultures were washed 3 times and dissolved in 1% SDS (250 μl). An aliquot (200 μl) was removed and added to scintillation fluid for counting. Values were normalized to control ^{35}S-cystine uptake (20 min exposure to ^{35}S-cystine without BMAA or CPG).

Glutathione assay

Total glutathione was assayed using a modification of a previous method (Baker et al., 1990; Lobner et al., 2003). Briefly, following exposure to BMAA for 3 h, cells were washed with a HEPES buffered saline solution, dissolved in 200 μl of 1% sulfosalicylic acid, and centrifuged. A 25 μl aliquot of the supernatant was combined with 150 μl of 0.1 M phosphate/5 mM EDTA buffer, 10 μl of 20 mM dithiobis-2-nitrobenzoic acid, 100 μl of 5 mM NADPH, and 0.2 U of glutathione reductase. Total glutathione was determined by kinetic analysis of absorbance changes at 402 nm for 1.5 min, with concentrations determined by comparison to a standard curve.

Assay of intracellular oxidative stress

Oxidative stress was assayed by measuring dichlorofluorescein oxidation using a fluorescent plate reader following a modification of a previous method (Wang and Joseph, 1999; Lobner et al., 2007). Cultures were exposed to 3 mM BMAA for 3 h in the presence of 5-(and -6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (10 μM). The carboxy-H_2DCFDA is de-esterified within cells to form a free acid that can then be oxidized to the fluorescent 2′7′-dichlorofluorescein (DCF). After the exposure to carboxy-H_2DCFDA, cultures were washed three times with culture media lacking serum. Fluorescence was then measured using a Fluoroskan Ascent fluorescence plate reader (ThermoLabsystems). The excitation filter was set at 485 nm and emission filter at 538 nm. Background fluorescence (no carboxy-H_2DCFDA added) was subtracted and the results normalized to control conditions (carboxy-H_2DCFDA added, but no BMAA exposure).

Analysis of glutamate release

Glutamate release was measured following exposure to 3 mM BMAA or cystine for 1 h. Experiments were performed in the presence of 100 μM DL-threo-β-benzyloxyaspartate (TBOA) to block reuptake of released glutamate by Na-dependent glutamate transporters and in the presence of 10 μM MK-801 to block potential injury induced glutamate release. Samples of the bathing media from the cell cultures were assayed for glutamate by using phenylisothiocyanate (PITC) derivatization, HPLC (Agilent 1100) separation using a Hypersil-ODS reverse phase column, and ultraviolet detection at a wavelength of 254 nm (Cohen et al., 1986; Lobner and Choi, 1996). 200 μl of the bathing media is derivatized with 100 μl of PITC, methanol, triethylamine (2,7,4) and dried under vacuum. These samples are then reconstituted in solvent consisting of 0.14 M sodium acetate, 0.05% TEA, 6% acetonitrile and brought to pH 6.4 with glacial acetic acid. The above solvent is used as the mobile phase with the column being washed between each sample run in 60% acetonitrile, 40%