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Role of glutamate receptors in tetrabrominated diphenyl ether (BDE-47) neurotoxicity in mouse cerebellar granule neurons

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HIGHLIGHTS

- Toxicity of BDE-47in cerebellar granule neurons is decreased by antagonists of ionotropic glutamate receptors.
- BDE-47 increases intracellular calcium and causes oxidative stress.
- These effects of BDE-47 appear to be secondary to an increase of extracellular glutamate.

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ABSTRACT

The polybrominated diphenyl ether (PBDE) flame retardants are developmental neurotoxicants, as evidenced by numerous *in vitro*, animal and human studies. PBDEs can alter the homeostasis of thyroid hormone and directly interact with brain cells. Induction of oxidative stress, leading to DNA damage and apoptotic cell death is a prominent mechanism of PBDE neurotoxicity, though other mechanisms have also been suggested. In the present study we investigated the potential role played by glutamate receptors in the *in vitro* neurotoxicity of the tetrabromodiphenyl ether BDE-47, one of the most abundant PBDE congeners. Toxicity of BDE-47 in mouse cerebellar neurons was diminished by antagonists of glutamate ionotropic receptors, but not by antagonists of glutamate metabotropic receptors. Antagonists of NMDA and AMPA/Kainate receptors also inhibited BDE-47-induced oxidative stress and increases in intracellular calcium. The calcium chelator BAPTA-AM also inhibited BDE-47 cytotoxicity and oxidative stress. BDE-47 caused a rapid increase of extracellular glutamate levels, which was not antagonized by any of the compounds tested. The results suggest that BDE-47, by still unknown mechanisms, increases extracellular glutamate which in turn activates ionotropic glutamate receptors leading to increased calcium levels, oxidative stress, and ultimately cell death.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been extensively used in recent years as flame retardants in a variety of consumer products. Since they are not chemically bound to the polymer product, they easily leach out into the environment. Levels of PBDEs in biota and in human tissues have significantly increased in the past three decades, and levels in North America are particularly high (USEPA, 2010; EFSA, 2011). There are 209 possible congeners of PBDEs; the most utilized penta- and octa-BDE mixtures have been banned in the E.U. and in several states in the U.S.A. Five congeners (BDE-47, -99, -100, -153, -154) predominate in human tissues, usually accounting for 90% of the total body burden, with BDE-47 (2, 2′, 4, 4′-tetrabrominated diphenyl ether) alone accounting for about 50% (USEPA, 2010).

Body burden of PBDEs is much higher (by 3–9-fold) in infants (because of exposure through breast milk), and in toddlers (because of exposure through house dust and the diet), raising concerns for their potential developmental toxicity and neurotoxicity (Costa and Giordano, 2007; Williams and DeSesso, 2010; Kodavanti et al., 2011; Dingemans et al., 2011). A plethora of animal studies indicate that prenatal and/or postnatal exposure to different PBDEs causes long-lasting behavioral abnormalities, particularly in the domains of motor activity and cognition (Branchi et al., 2002; Dufault et al., 2005; Viberg et al., 2003;

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Cheng et al., 2009; Kodavanti et al., 2010; Ta et al., 2011). Animal evidence is supported by human studies which are suggestive of neurodevelopmental effects of PBDEs, including poorer attention, decreased motor coordination and cognition, altered adaptive behavior, and increased activity/impulsivity behaviors (Herbstman et al., 2010; Chao et al., 2011; Shy et al., 2011; Eskenazi et al., 2013).

The mechanisms of PBDEs' developmental neurotoxicity have not been fully defined. However, two general modes of action have emerged, one indirect, mediated by interference of PBDEs with thyroid hormones, and the other involving direct effects of PBDEs on the developing brain (Costa and Giordano, 2007; Alm and Scholz, 2010; Costa et al., 2014). There is evidence that PBDEs, possibly through their hydroxylated metabolites, may alter thyroid hormone homeostasis, and this would undoubtedly contribute to adverse neurodevelopmental effects; however, there is also evidence of developmental neurotoxicity of PBDEs in the absence of alterations in thyroid hormones (Gee et al., 2008; Kim et al., 2012; Costa et al., 2015). Evidence abounds indicating that PBDEs directly affect brain cells. PBDEs induce oxidative stress in various brain cells system *in vitro* (He et al., 2008; Costa et al., 2010a; Tagliaferri et al., 2010; Giordano et al., 2008), as well as *in vivo* (Cheng et al., 2009; Costa et al., 2015). Oxidative stress may result from the ability of PBDEs to disrupt mitochondria, though the exact molecular mechanisms need further scrutiny. The downstream effects of PBDE-induced oxidative stress are DNA damage (He et al., 2008; Gao et al., 2009; Pellacani et al., 2012) and apoptotic cell death (Giordano et al., 2008; He et al., 2008; Tagliaferri et al., 2010; Costa et al., 2015). In addition, a variety of other potential mechanisms have also been investigated in *in vitro* and *in vivo* studies across different PBDE congeners. Some relate to effects on signal transduction, others to receptor and neurotransmission modulation, mostly related to effects on the cholinergic, dopaminergic, GABAergic, and glutamatergic systems (Costa et al., 2014).

The present study was prompted by the findings of Reistad et al. (2006, 2007) who showed that *in vitro* cytotoxicity of DE-71 (a pentaBDE mixture) and that of tetrabromobisphenol-A (TBBPA; a brominated flame retardant) in rat cerebellar neurons was reduced by MK-801, an antagonist of the *N*-methyl-D-aspartate (NMDA) receptor. Findings with DE-71 were later confirmed by Yu et al. (2008) in human neuroblastoma cells. The aim of the present study was to further explore the role of glutamate receptors in modulating the neurotoxicity of BDE-47 *in vitro* and to explore potential underlying mechanisms.

2. Materials and methods

2.1. Materials

BDE-47 (99.9%) was purchased from AccuStandard Inc. (New Haven, CT). The glutamate receptor antagonists 2,3-dihydroxy-6-nitro-sulfamoylbenzo [f] quinoxaline (NBQX), (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (2S)-2-amino-2-[(1S,2S)-2-carboxycy cloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), and (RS)-α-methylserine-*O*-phosphate (MSOP) were from Tocris Cookson (Ellisville, MO), while (+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d] cyclohepten-5,10-iminemaleate (MK-801) was from Sigma–Aldrich (St. Louis, MO). Neurobasal-A medium, gentamycin, fetal bovine serum and 5-(and-6)-carboxy-2′-7′-dichloro-fluorescein diacetate were from Invitrogen (Carlsbad, CA). The protein bicinchoninic acid assay was from Thermo Fischer Scientific (Rockford, IL). Anhydrous dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and all other chemicals were from Sigma–Aldrich, while the Thiobarbituric Acid Reactive Substances (TBARS) assay kit was purchased from Cayman Chemical Company, Ann Arbor, MI.

2.2. Cell culture

Cultures of cerebellar granule neurons (CGNs) were prepared from 7-day-old C57Bl/6J mice killed by decapitation after CO_2 narcosis, as described in detail by Giordano et al. (2006, 2008). After removal of meninges from the whole brain, cerebella were rapidly dissected and cut into small cubes. The matrix was digested at 37 °C for 30 min in 1.5 mg/ml papain solution. After addition of HBBS buffer, tissue was sedimented by centrifugation, the supernatant carefully removed, and the cerebellar tissue was re-suspended in HBBS buffer. The tissue was mechanically dissociated using a long-stem Pasteur pipette and the cell suspension was centrifuged at 900 × *g* for 5 min. The cell pellet was re-suspended in complete growth medium consisting of Neurobasal A medium containing 1.5 mM GlutaMAX, 250 mg/ml fungizone, gentamycin, KCl 26 mM, and B27, a medium supplement with a newly improved formulation that substitutes serum. Neurons were seeded in poly-D-lysine-coated 48-well plates at the density of $5 \times 104/cm^2$, and cultured for 10 days before treatments. Four days after seeding, neurons were treated with cytosine arabinofuranoside (AraC; 3 μM) in complete Neurobasal A medium, containing B27 minus AO (antioxidants) to prevent glial proliferation and obtain neuron-enriched cultures. Four days after AraC treatment, 50% of medium was replaced with fresh complete medium.

2.3. Cell treatments

BDE-47 was dissolved in DMSO to obtain stock solution of 12.5 mM, which was diluted appropriately at the time of use in medium-B27 minus AO. The ionotropic or metabotropic glutamate receptor antagonists and BAPTA-AM were added 30 min before BDE-47.

2.4. Cytotoxicity assay

Cell viability was quantified using the metabolic dye MTT, as previously described (Giordano et al., 2006, 2008). Briefly, cells were treated with BDE-47 for 24 h at 37 °C. At the end of exposure, the medium was removed and cells were incubated with 500 μl/well of Locke's buffer solution containing 2 mg/ml MTT for 30 minutes. MTT was then removed and the reaction product was dissolved in 0.25 ml DMSO/well. Absorbance was read at 570 nm, and the results expressed as the percentage of viable cells relative to the DMSO exposed controls. Untreated controls and blanks were incubated in the same plates and under the same conditions.

2.5. Measurement of glutamate release

Extracellular glutamate was measured essentially as described by Giordano et al. (2006). CGN were exposed to BDE-47 for various times (5–60 min), after which supernatants were collected, and cells were scraped to determine protein content. Determination of L-glutamate was carried out using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay kit (Molecular Probes, Invitrogen). The kit is designed for detecting glutamic acid by virtue of its oxidation by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. The latter reacts with Amplex Red reagent in a 1:1 stoichiometric reaction catalyzed by horseradish peroxidase to generate the fluorescent product, resorufin. Fluorescence was measured at 560 and 590 nm for excitation and emission, respectively. A standard curve with glutamate indicated linearity between 20 nM and 10 μM.