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Original research article

TBBPA causes neurotoxic and the apoptotic responses in cultured mouse hippocampal neurons *in vitro*

Konrad A. Szychowski, Anna K. Wójtowicz *

Department of Animal Biotechnology, Animal Sciences Faculty, University of Agriculture, Kraków, Poland

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ABSTRACT

Background: Tetrabromobisphenol A (TBBPA) is a brominated flame retardant widely used in a variety of commercial and household products. TBBPA can become bioaccumulated in human body fluids, and also in different brain regions. The aim of the present study was to determine the viability and apoptosis of cultured mouse hippocampal neurons *in vitro* after exposure to TBBPA. Additionally, we examined the involvement of ROS generation in the effect of TBBPA.

Methods: Primary hippocampal neuron cultures were prepared from Swiss mouse embryos on day 17/18 of gestation. The cultures were treated with TBBPA at concentrations ranging from 1 nM to 100 μM for 30 min or 3, 6 or 24 h. To study apoptosis, the activity of caspase-3 was measured, and apoptotic body formation was evaluated. To investigate the cytotoxic effect of TBBPA, the level of lactate dehydrogenase (LDH) was measured in the culture medium.

Results: Our results demonstrated that TBBPA concentrations ranging from 100 nM to 100 μM caused caspase-3 activation and apoptotic body formation. The cytotoxic effects of TBBPA were observed at concentrations ranging from 50 nM to 100 μM. To detect intracellular ROS, the fluorogenic dye H2DCFDA was used. We did not observe any significant increase in the level of cellular ROS in cultured cells after TBBPA treatment. However, in a cell-free model, TBBPA at concentrations ranging from 10 to 100 μM interacted with H2DCFDA and enhanced the fluorescence signal.

Conclusion: We suggest that the H2DCFDA assay cannot be used to measure TBBPA-stimulated cell-mediated ROS production.

Introduction

Tetrabromobisphenol A (TBBPA) is a brominated flame retardant (BFR) widely used in a numerous variety of commercial and household products, such as plastics, textiles and electronic appliances, including computers and televisions [1]. Sellström and Jansson [2] showed that TBBPA can leak from products and, due to its lipophilicity and environmental stability, can become bioaccumulated in living organisms [3]. TBBPA has been detected in human body fluids such as breast milk (1.8 ng/g lipid weight (lw) (≈3.32 nM)), blood (37.34 ng/g lw (≈69 nM)), and cord serum (649 ng/g lw (≈1 μM)) [4–7]. In addition, TBBPA accumulates in different brain regions and induces behavioral alterations in a mouse model *in vivo* [8,9]. These data suggest that humans chronically exposed to TBBPA may contain TBBPA in the brain at concentrations potentially even higher than those in body fluids. However, information about the neurotoxicity of this compound is very limited.

The available data showed TBBPA at low-μM concentrations caused lactate dehydrogenase (LDH) release, stimulated caspase-3 activity and induced DNA fragmentation in primary rat cerebellar granule cells and mouse cortical neurons *in vitro* [10–12]. The mechanism of TBBPA action in the nervous system appears to be complex. It is hypothesized that this compound can act *in vitro via* different molecular pathways, including the involvement of the N-methyl-D-aspartate (NMDA) receptor, the γ-aminobutyric acid (GABA) receptor, and peroxisome proliferator-activated receptor gamma (PPAR-γ) and either a direct or indirect increase in the intracellular calcium level [10–13]. The activation of all of these processes leads to an increase in the amount of cellular reactive oxygen species (ROS). Currently, it is essential to understand the mechanism of TBBPA action using a cell culture model to determine the involvement of ROS generation in this process.

ROS is a commonly used term that includes not only oxygen radicals (superoxide and hydroxyl) but also some non-radical

* Corresponding author.
E-mail addresses: konrad.szychowski@gmail.com (K.A. Szychowski), anna.wojtowicz@ur.krakow.pl (A.K. Wójtowicz).

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derivatives of molecular oxygen (O_2) such as hydrogen peroxide (H_2O_2) [14]. The overproduction of ROS disrupts physiological cellular homeostasis and results in apoptosis *via* the activation of the mitochondrial apoptosis pathway [15], which is a very common effect of exposure to many environmental xenobiotics. An assay that employs the substrate 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is the most popular method used to measure the ROS level in different cultured cell types due to its sensitivity.

The hippocampus is a major component of all mammalian brains that plays an important role in the consolidation of information from short-term memory to long-term memory and in spatial navigation. Hippocampal cells *in vitro* are among the most sensitive cultured cell types to different stimuli [16,17]. Due to the sensitivity of hippocampal neurons to different stimuli, this cell type appears to serve as a good model to determine the involvement of ROS production in the effects of TBBPA exposure on cultured cells.

Therefore, the aim of the present study was to determine the viability and apoptosis of mouse hippocampal neurons after 6 h of exposure to TBBPA. Additionally, we examined the involvement of ROS generation in the effects of TBBPA.

Materials and methods

Reagents

Neurobasal medium without phenol red and B27-AO supplement were purchased from Life Technologies (Grand Island, NY, USA). Trypsin, charcoal/dextran-treated fetal bovine serum (FBS), penicillin, streptomycin, staurosporine, TBBPA, H_2DCFDA and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The LDH-based cytotoxicity detection kit was purchased from Roche Applied Science (Mannheim, Germany). PBS was purchased from BIOMED (Lublin, Poland). Caspase-3 substrate was purchased from Calbiochem (Merck Corporation, Darmstadt, Germany). Stock solutions were prepared by dissolving the compounds in DMSO. TBBPA and other reagents were dissolved in DMSO. The final DMSO concentration in the culture medium was always equal to 0.01%.

Primary hippocampal cell cultures

The primary cultures of hippocampal neurons were prepared from the fetuses of pregnant female Swiss mice as previously described in detail [18–20]. Brain tissues were collected from mouse fetuses on day 17 or 18 of gestation. Pregnant females were anesthetized using CO_2 vapor and sacrificed *via* cervical dislocation. All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Bioethics Commission (no. 83/2012) in compliance with Polish law. The brains were removed from the fetuses, and the hippocampal tissues were dissected. The dissected tissue was minced into small pieces and then gently digested with trypsin. Then, the cells were centrifuged, and the pellet was resuspended in phenol red-free Neurobasal medium supplemented with 5% FBS. The cells were plated on poly-L-ornithine-coated (0.01 mg/mL) multi-well plates. After 2 days, the culture medium was replaced with phenol red-free Neurobasal medium supplemented with B27-AO (2 μL/mL), glutamine (2 mM), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, which is recommended for primary neuronal cultures [18,19]. This procedure typically yields cultures that contain approximately 90% neurons and 10% astrocytes [21]. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and were cultivated for 7 days *in vitro* prior to the experiment. The culture medium was changed prior to treating the cultures with the indicated compounds for this study.

Experimental treatment of the cultured hippocampal neurons in vitro

Primary hippocampal neuron cultures were exposed to 1 nM–100 μM TBBPA for 30 min or 3, 6 or 24 h. For the cytotoxicity and apoptosis assays, the cells were plated on 96-well plates at a density of 1.8×10^5 cells per cm^2. Controls with or without DMSO vehicle were included in the experimental design to determine the effect of DMSO (results not shown). After 6 h in culture, 100 μL of the medium was collected for the LDH assay, and the cells were collected and frozen at −80 °C for the caspase-3 activity measurements. To measure the generation of ROS, the cells were seeded on black-sided, clear-bottomed 96-well plates and exposed to TBBPA at the indicated experimental concentrations for 30 min or 3 or 24 h. For Hoechst 33342 and calcein AM staining, the neurons were seeded on polyornithine-coated coverslips placed in 24-well plates and were initially cultured for 7 days to allow for differentiation; then, the neurons were exposed to TBBPA for 6 h. The aim of the present study was to investigate the effect of TBBPA on the viability and apoptosis of cultured mouse neocortical neurons *in vitro*.

LDH cytotoxicity assay

The cytotoxicity detection kit is a colorimetric assay for the quantification of cell death and cell lysis based on the release of LDH from the cytosol of damaged cells into the surrounding medium [22]. An increase in the amount of dead or plasma membrane-damaged cells results in an increase in LDH activity in the culture medium. After 6 h of treatment with 1 nM–100 μM TBBPA, 100 μL of the culture supernatants were collected and incubated in the reaction mixture from the kit. After 30 min, the reaction was stopped by adding 1 N HCl, and the absorbance at a wavelength of 490 nm and a reference wavelength of 600 nm was measured using the ELISA microplate reader manufactured by Bio-Tek Instruments (Biokom). The results are expressed as the mean percentages ± SEM relative to the control from eight separate samples, and the samples were measured in quadruplicate.

Hoechst 33342 and calcein AM staining

Apoptotic cells exhibit nuclear condensation and DNA fragmentation, which can be detected *via* vital staining using Hoechst 33342. Hoechst 33342 binds to DNA fragments and apoptotic bodies, emitting blue fluorescence, whereas living cells exhibit esterase activity that is visualized as green-fluorescent light *via* calcein AM staining. Therefore, this staining method is used to indicate metabolic activity and cell viability [16]. Hippocampal neurons at 7 days *in vitro* were exposed to 1 μM TBBPA, and the cells were cultured for an additional 6 h. After this period, the cells were washed with phosphate-buffered saline (PBS) and exposed to Hoechst 33342 and calcein AM diluted in PBS and added to the medium at a final concentration of 10 and 4 μM, respectively. The cells were incubated for 15 min in an atmosphere of 5% CO_2/95% air at 37 °C, washed one time in PBS and visualized using a fluorescence microscope (Nikon, Japan).

Caspase-3 activity

Caspase-3 activity was used as a marker of cell apoptosis and was assessed according to Nicholson et al. [23]. Cultured neurons were lysed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT) in 10 °C for 10 min. The lysates were incubated in the caspase-3 substrate