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Tetrabromobisphenol-A induces apoptotic death of auditory cells and hearing loss

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ABSTRACT

Phenolic tetrabromobisphenol-A (TBBPA) and its derivatives are commonly used flame-retardants, in spite of reported toxic effects including neurotoxicity, immunotoxicity, nephrotoxicity, and hepatotoxicity. However, the effects of TBBPA on ototoxicity have not yet been reported. In this study, we investigated the effect of TBBPA on hearing function *in vivo* and *in vitro*. Auditory Brainstem Response (ABR) threshold was markedly increased in mice after oral administration of TBBPA, indicating that TBBPA causes hearing loss. In addition, TBBPA induced the loss of both zebrafish neuromasts and hair cells in the rat cochlea in a dose-dependent manner. Mechanistically, hearing loss is largely attributed to apoptotic cell death, as TBBPA increased the expression of pro-apoptotic genes but decreased the expression of anti-apoptotic genes. We also found that TBBPA induced oxidative stress, and importantly, pretreatment with NAC, an anti-oxidant reagent, reduced TBBPA-induced reactive oxygen species (ROS) generation and partially prevented cell death. Our results show that TBBPA-mediated ROS generation induces ototoxicity and hearing loss. These findings implicate TBBPA as a potential environmental ototoxin by exerting its hazardous effects on the auditory system.

1. Introduction

Brominated flame retardants (BFRs) are a large group of compounds widely used to protect various products, such as plastics, textiles, and electronic equipment from catching fire. These compounds may bioaccumulate and pose an environmental health problem. Phenolic tetrabromobisphenol-A (TBBPA) is one of the most important BFRs used in industry [1].

TBBPA is a phenolic compound that is primarily used as a chemical flame retardant, although it has low emission into the environment, TBBPA may leak from treated products and be absorbed through the skin in humans [2]. Thomsen et al. reported the presence of TBBPA in the blood serum of electronic dismantlers, circuit board producers, and laboratory personnel [3]. TBBPA is potentially neurotoxic [4] and may disrupt estrogen homeostasis *in vitro* and *in vivo* [5,6]. TBBPA also induces polycystic lesions in the kidneys of exposed newborn rats [7] and suppresses the induction of interleukin-2 in murine splenocytes *in vitro*, indicating potentially immunotoxicity [8]. In zebrafish, TBBPA increases formation of reactive oxygen species (ROS), resulting in an oxidative stress response and/or apoptosis [9]. However, the effect of TBBPA on hearing function has not yet been reported.

Various types of stressors, such as aging, noise, drugs, infection, or immune-induced inflammation, may result in damage to the hair cells in the cochlea, leading to hearing impairment. Hair cells, the sensory receptors for the auditory system, are extremely susceptible to injury, and mammalian hair cells cannot regenerate, unlike the hair cells of aquatic and avian animals [10]. It is well established that ototoxic drugs, such as aminoglycoside antibiotics and anti-cancer drugs (e.g., cisplatin) [reviewed in [11,12]] generate

Abbreviations: ABR, auditory brainstem response; BFR, brominated flame retardants; RNS, reactive nitrogen species; ROS, reactive oxygen species; TBBPA, phenolic tetrabromobisphenol-A.

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free radicals, including ROS and reactive nitrogen species (RNS), that cause apoptotic cell death [reviewed in [13,14]]. Agents that scavenge ROS or block their formation have been proposed to protect the inner ears against such stressors [15–20].

In this study, we investigated the effect of TBBPA on hearing function by analyzing hair cells in the rat cochlea and zebrafish neuromasts. In addition, we examined apoptotic cell death, measured ROS levels, and evaluated the protective effect of an antioxidant against TBBPA-induced hearing loss.

2. Materials and methods

2.1. Cell culture and animals

The establishment and characterization of the HEI-OC1 auditory cell line was previously described by Kalinec et al. [21]. HEI-OC1 cells were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 U/ml interferon-γ (Genzyme, Cambridge, MA, USA), and cultured at 33 °C with 5% CO_2. Sprague-Dawley (SD) rats and ICR mice were purchased from Orient Bio Inc. (Seongnam, Korea). Animal studies were approved by the Animal Care and Use Committee at Wonkwang University (WKU16-1). Wild type zebrafish were maintained at 28.5 °C on a 14 h light/10 h dark cycle. For hair cell staining, 5-day old zebrafish (n = 20) were treated with TBBPA added directly to the EM. The hair cell lateral line neuromasts were labeled using 2.5 μM Yo-Pro1 (Molecular Probes, Eugene, OR, USA) for 30 min followed by three rinses with EM. Zebrafish were anesthetized with 8 μg/mL MS-222 (Sigma Chemical Co., Saint Louis, MO, USA) and mounted with methylcellulose in a depression slide for observation under a fluorescence microscope.

2.2. Drug treatment

N-acetyl-cysteine (NAC), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), and gelatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Culture wares were purchased from Falcon, Inc. (Becton Dickinson Biotech, IL, USA). DMEM, FBS, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), hydroethidine (HE), and Yo-Pro1 were obtained from Invitrogen (Invitrogen, CA, USA). Antibodies against caspase-3, Bcl-2, Bak, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cells (5×10^4 cells/well of 24-well plates) or cochlear hair cells were incubated with Tetrabromobisphenol-A (TBBPA, Sigma-Aldrich, St. Louis, MO, USA) for the indicated time. To determine cell viability, 1 ml of cell suspension was treated with 0.5 mg of MTT (Sigma-Aldrich, Saint Louis, MO, USA) for 3 h and washed three times with phosphate buffered saline (PBS, pH 7.4). The insoluble formazan product was dissolved in DMSO and the optical density (OD) of each well was measured using a microplate reader (Titertek Multiskan, Flow Laboratories, McLean, VA, USA) at 590 nm. ICR mice weighing 18–20 g were randomly divided to two groups: a control group (n = 10) and mice treated with 250 mg/kg TBBPA (n = 10) for 30 days. Animals were fed a standard commercial diet, while housed at an ambient temperature of 20–22 °C, a relative humidity of 50 ± 5%, and under a 12 h light/12 h dark cycle in a pathogen-free facility.

2.3. Auditory brainstem response

Auditory brainstem response (ABR) was measured by System-3 hardware and software (Tucker Davis Technologies, Alachua, FL), with 1000 stimulus repetitions per record. 8 week-old ICR mice were anesthetized using a mixture of ketamine (40 mg/kg) and xylazine (10 mg/kg) and kept warm during ABR recording. A subdermal needle electrode was inserted at the vertex, while ground and reference electrodes were inserted subdermally into the loose skin beneath the pinnae of the opposite ear. Tone bursts of 4 ms durations and a rise-fall time of 1 ms at frequencies of 4, 8, 16, and 32 kHz and a click were presented to the right and left ear through an inserted speculum in the external auditory meatus. The sound intensity varied in 5 dB intervals near the threshold.

2.4. Organotypic cultures of Corti organ explants

SD rats were sacrificed on postnatal day 2 (P2), and the temporal bones were isolated in a sterile manner. After placing the tissue in a 6 cm dish with ice-cold PBS, the cochlear capsule was peeled away and the membranous labyrinth was exposed. The spiral ligament and stria vascularis were removed and the organ of Corti was dissected under a microscope. Each explant was placed onto a 0.1% gelatin-coated glass coverslip in a 4-well dish containing DMEM supplemented with 10% FBS. Culture wells, each containing 500 μl of medium, were maintained in an incubator at 37 °C for 16 h with 5% CO_2 and 95% humidity.

2.5. Measurement of intracellular ROS

Cells were treated with 10 μM DCFH-DA and were further incubated for 1 h at 37 °C. The fluorogenic oxidation of HE to ethidium (E+) has been used as a measure of O_2^-. The cells were rinsed with PBS and detected through flow cytometric analysis. Fluorescence intensity was measured using a BD FACSCalibur flow cytometer. For each analysis 20,000 events were collected and analyzed using the FL1 (DCFH-DA) channel and FL2 (HE) channel. Data were analyzed using BC Cell Quest software.

2.6. Western blotting and reverse transcription (RT)-PCR

Western blotting and RT-PCR were performed as previously reported [20]. Briefly, western blotting used explants or HEI-OC1 cells lysed and boiled for 15 min. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight at 4 °C with primary antibodies (1:1000) followed by an anti-rabbit or anti-goat IgG antibody conjugated to horseradish peroxidase (HRP) (1:2000) for 1 h. The immunoreactive signal was detected using an enhanced chemiluminescence (ECL) detection system. Expression levels of each enzyme were normalized by the respective level of β-actin. For RT-PCR, cDNAs synthesized from total RNAs of whole cochlea were used for PCR using gene-specific primers listed as follows: IL-6 (forward: 5′-TTGCCTTCTTGGGACTGATGC-3′, reverse: 5′-TTGGAAATTGGG GTAGGAAGGA-3′), and β-actin (forward: 5′-AGACAGCCGCATCTTCTTGTC-3′, reverse: 5′-CCACAGTCTTCTGAGTGGCAC-3′). The amplified PCR products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV.

2.7. Caspase activity assay

An equal amount of total proteins prepared from whole-cell lysate was quantified using a BCA protein quantification kit (Sigma) in each lysate. Catalytic activity of caspase-3 from cell lysate was measured by proteolytic cleavage of 100 μM 7-amino-4-methylcoumarin (AMC)-DEVD, a fluorogenic substrate (Calbiochem, San Diego, CA, USA), for 1 h and AMC as a negative standard.

2.8. Phalloidin staining

Cochlear explants were fixed with 4% paraformaldehyde (PFA)

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