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Toxicity of TDCPP and TCEP on PC12 cell: Changes in CAMKII, GAP43, tubulin and NF-H gene and protein levels

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HIGHLIGHTS

- The toxicity of TDCPP/TCEP in undifferentiated and differentiated PC12 cells was modelled.
- Both cytotoxicity and neurotoxicity were elicited by TDCPP/TCEP.
- CAMKII, GAP43, tubulin and NF-H maybe useful biomarkers for the cytotoxicity and neurotoxicity.

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ABSTRACT

TDCPP and TCEP are two major types of organophosphorus flame retardants (OPFRs) that are bioaccumulative and persistent in the environment. The toxicity effects of TDCPP and TCEP on PC12 cell are not well understood. In the present study, we investigated morphology, viability and apoptosis in cultured PC12 cells in response to TDCPP and TCEP. The mRNA and protein expression levels of CAMKII, GAP43, tubulin and NF-H were quantified in PC12 cells treated with varying concentrations of the two agents. Results indicate that, upon treatment with the two OPFRs, cell growth decreased, apoptosis increased, morphology was altered and significant changes were found in the gene and protein levels. Treatment with TDCPP caused a reduction in the levels of each of the six proteins studied and in the gene levels of GAP43, NF-H and the two tubulins, but it resulted in an increase in CAMKII gene levels. Treatment with TCEP resulted in similar changes in gene levels to TDCPP and led to decreases in the protein levels of GAP43 and the tubulins while increasing the CAMKII and NF-H protein levels. These results suggest that changes in the gene and protein levels of the regulatory proteins (CAMKII, GAP43) and the structural proteins (tubulin, NF-H) are due to different mechanisms of the toxins, and these proteins may be useful biomarkers for the cytotoxicity and neurotoxicity.

1. Introduction

Because flame retardants (FRs) have the ability to slow the combustion of treated materials, many varieties of FRs have been added to the polymers and resins that can be found in electronics, furniture and textiles. Polybrominated diphenyl ethers (PBDEs) have been the most commonly used FRs in the past several decades (IPCS, 1998; Sjödin et al., 2003; Stapleton et al., 2009). However, an increasing line of evidence suggests that PBDEs have potential adverse environmental and human health effects. Adrian Covaciet et al. have reported that PBDEs have a tendency to leach out of treated products into the environment, and their bioaccumulative and persistent qualities have resulted in a more ubiquitous detection of PBDEs. PBDEs can be detected even in human milk, resulting in decreased weight and cryptorchidism in the neonatus (Covaci et al., 2011; Stapleton et al., 2008; Herbstman et al., 2010). All of the above suggest that the discovery of a replacement to reduce or cease the applications of PBDEs is necessary. In 2004, the US halted the production of PentaBDE because of the more generalised use of organophosphorus flame retardants (OPFRs), one of the replacements of PBDEs (van der Veen and de Boer, 2012).

However, some doubt remains about the safety of OPFRs. The OPFRs consist of tris-(2-chloroethyl) phosphate (TCEP), tris-2-chloroisopropyl phosphate (TCPP), tris-(1,3-dichloro-2-propyl)

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phosphate (TDCPP), tris-(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPP), tricresyl phosphate (TCP) and others. These compounds have been detected in indoor dust, furniture, outdoor air, soil and sediment worldwide (Andresen et al., 2004; Stapleton et al., 2009). For instance, Cao et al. and Wang et al. collected water from the Songhua River and sediments from Taihu Lake in China, respectively, and found that the concentrations of TDCPP were 2.5–40 ng/L and 0.62–5.54 μg/kg (Wang et al., 2011; Cao et al., 2012). In the US and Japan, the concentration of TDCPP in dust samples is similar to the concentrations of PBDE (Stapleton et al., 2009). A survey of Europe's sewage treatment plant water quality illustrated that the detected concentrations of TCEP in the majority of plants fall in the range of several hundred ng/L (Reemtsma et al., 2006). In addition, research scholars have found an accumulation of OPFRs in the fish of Switzerland and in the ova of Herring gulls in Filipino Lake (Rodil et al., 2009). Because of its use in a variety of decoration materials and plastic products, the densities of OPFRs in indoor environments, such as the bedroom, office and car, are hundreds-fold higher than that of outdoor environments. The concentrations of OPFRs of indoor air range from 10 ng/m^3 to 100 ng/m^3, and in Switzerland, TCEP's concentration was the highest in indoor air samples (Staaf and Östman, 2005).

This developing evidence proves that OPFRs have the same bioaccumulative and persistent environmental qualities as PBDEs, and these qualities may result in similar damages and effects at the ecological, environmental and human health levels. Although many studies have been performed on the toxic effects of OPFRs, including TDCPP and TCEP (Farhat et al., 2014; Föllmann and Wober, 2006), data on the mechanisms of these toxic effects are limited (Dishaw et al., 2011). OPFRs exhibit a similar structure to organophosphorus (OP) pesticides, which are known to cause neurotoxicity, indicating that OPFRs may exert similar neurotoxicity actions. Studies have shown that TDCPP could cause neurodevelopmental toxicity, endocrine disruption and even genotoxicity and carcinogenicity (IPCS, 1998; Meeker and Stapleton, 2010). Furthermore, results of a study that use PC12 cells as model suggested that TDCPP affects the synthesis of DNA in a similar manner to OP pesticides (Dishaw et al., 2011).

In the present study, we investigated the toxic effects of two major OPFRs, TDCPP and TCEP, using the PC12 cell line, a widely used in vitro model for neurotoxicity. This study aims to observe the changes in cell morphology under the stimulation of TDCPP and TCEP to investigate the effects of various concentrations of TDCPP and TCEP on cell viability and apoptosis. To further explore which proteins are possible markers of the toxicity and the possible mechanisms of the effects of TDCPP and TCEP on PC12 cells, we used western blotting and fluorescence quantitative PCR to detect changes in protein synthesis and mRNA expression of related proteins, including CAMK2A/CAMK2B (calcium (Ca^{2+})/calmodulin dependent kinase type II), GAP43 (growth associated protein-43), tubulin-alpha(α), tubulin-beta(β) and NF-H (neurofilament).

2. Materials and methods

2.1. Cell cultures

Experiments were performed on cells that had undergone fewer than five passages. PC12 cells (the rat pheochromocytoma cell line) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Gibco-BRL, USA) supplemented with 10% inactivated horse serum (Gibco-BRL, USA), 5% inactivated foetal bovine serum (Gibco-BRL, USA) and 50 μg/ml penicillin streptomycin (Invitrogen, USA). To induce differentiation of the PC12 cells, 50 ng/ml nerve growth factor (NGF) (Invitrogen, USA) was added. The cells were incubated with 5% CO_2 at 37 °C, standard conditions for PC12 cells.

To study the effects of TDCPP and TCEP on the undifferentiated PC12 cells, the culture medium was changed to include different concentrations of TDCPP (Sigma, USA) or TCEP (Sigma, USA) after seeding for 24 h. After incubating in the two test agents for six days, the culture medium was again changed to include 50 ng/ml NGF. To study the effects of the compounds in differentiating cells, the same test agents were added at the start of NGF treatment. The media, along with the indicated agents, was changed every 48 h. Control groups received 0.1% DMSO (v/v) (Sigma, USA). All of the cultures, before and after treatment with NGF, TDCPP or TCEP, were examined under an inverted phase contrast microscope to examine cell morphology.

2.2. Cell viability

After treatment with TDCPP and TCEP, the cell viability was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and experiments were performed in independent groups of each test compound assay. The concentrations of TDCPP and TCEP were 1, 5, 10, 15, 20, 25 and 50 μM and 15, 20, 40, 80, 150, 200 and 400 μM, respectively. Each assay included a group as a blank control. Briefly, PC12 cells were suspended at a final concentration of 1.2×10^5 cells/well, were cultured in 96-well culture plates and were incubated with the test compounds for the indicated time period. Following incubation, 10 μl of the MTT solution (5 mg/ml) was added into each well at a final concentration of 0.5 mg/ml, and the plates were then incubated for an additional 4 h. After the medium was removed, DMSO (dimethyl sulphoxide) (150 μl) was added into each well before reading the microplates at 490 nm. Cell viability was expressed as a percentage of the control culture value.

2.3. Analysis of flow cytometry

After the cells were incubated in 5, 15, 25 and 50 μM of TDCPP or 40, 80, 150 and 200 μM of TCEP, flow cytometry (FCM) was used to detect levels of apoptosis using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Blank and solvent control groups were included. Cells were collected and centrifuged. Then, the centrifuged cells were rinsed twice in PBS and suspended with Binding Buffer at a concentration of 1×10^6 cells/ml; the resuspended cells were subsequently incubated for 15 min with 5 μl FITC and 5 μl PI for every 1×10^5 cells at RT in the dark. To detect apoptosis, 400 μl Binding Buffer was added to the cells again, and the cells were then analysed with a FACScan flow cytometre (BD.FACS Calibur, USA).

2.4. Real time qPCR

To determine the effects of TDCPP and TCEP on mRNA expression, groups treated with 15, 25 and 50 μM of TDCPP and 40, 80, 150 and 200 μM of TCEP. We used RT-qPCR to detect alterations in the mRNA expressions of CAMKII, GAP43, tubulins and NF-H. Isolation of total RNA, synthesis of first-strand cDNA and RT-PCR were performed as previously described (Yu et al., 2010). Total RNA was isolated from PC12 cells using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's recommendations. To remove genomic DNA contamination, RNase-free DNaseI (Sigma, USA) was used. The purity and quality of the RNA were determined by measuring 260/280 nm ratios and by 1% agarose–formaldehyde gel electrophoresis with ethidium bromide staining. Synthesis of first-strand cDNA was performed using a Prime Script RT Reagent Kit (TaKaRa, Shanghai, China) following the manufacturer's instructions. The RT-qPCR was performed using SYBR Real-time PCR Master-Mix-Plus kits (Toyobo, Osaka, Japan) and was analysed on an ABI 7300 System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The primer sequences of genes are shown in Table 1. The amplification protocol was as follows: initial denaturation for 30 s at 95 °C, followed by 40 cycles of 95 °C for 40 s and 60 °C for 31 s.

2.5. Western blot analysis

PC12 cells were cultured on six-well plates and subjected to the same treatment as the above groups. Cells were lysed in lysis buffer on ice for 20 min, and a protease inhibitor cocktail was added. Then, the lysates were clarified by centrifugation at 4 °C for 10 min at 14,000 g. After quantifying the protein concentration with a BCA protein assay, the lysates were separated by 8% SDS-PAGE (30 μg of protein/lane)

Table 1
Primer sequences for test genes.

Gene name	Primer sequence (5′-3′)	Length (bp)
Tubb3	Forward: 5′-CAGATGCTGGCCATTCAGAGTAAG-3′ Reverse: 5′- TGTTGCCGATGAAGGTGGAC-3′	127
Tuba	Forward: 5′-GATGCAGCCAATAATTATGCCAGAG-3′ Reverse: 5′-TCCATCAGCAGAGATGCAAACC-3	173
NF-H	Forward: 5′- AAGGAAACCGTCATTGTAGAGGAA-3′ Reverse: 5′- GGAGACGTAGTTGCTGCTTCTT-3′	172
GADPDH	Forward: 5′-CCTTCATTGACCTCAACTACATG -3′ Reverse: 5′- CTTCTCCATGGTGGTGGAAAC-3′	
CAMK2a	Forward: 5′- GATGTGCGACCCTGGAATGA -3′ Reverse: 5′- ATGTAGGCGATGCAGGCTGAC -3′	183
CAMK2b	Forward: 5′-CCCATGGACACAGGGTAAACATC -3′ Reverse: 5′- GCCTCACGCTGTCTGTGACAA -3′	90
GAP43	Forward: 5′- CCGACAGGATGAGGGTAAAG -3′ Reverse: 5′- GCAGGAGAGACAGGGTTC -3′	197