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The involvement of autophagy and cytoskeletal regulation in TDCIPP-induced SH-SY5Y cell differentiation

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ABSTRACT

Exposure and toxicity to organophosphate-based flame retardants are an increasing health concern. Neurons appear to be particularly vulnerable to the effects of these chemicals. For example, in vitro studies have shown that tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) induces apoptosis and autophagy in neural cells. In the present study, we investigated the cell biological mechanisms of TDCIPP-induced neurotoxicity using undifferentiated human SH-SY5Y neuroblastoma cells as a model. Interestingly, TDCIPP treatment promoted differentiation in SH-SY5Y cells, which displayed various alterations including neurite elongation, an expansion of the numbers of neurite-bearing cells, and an increase in expression of cytoskeletal components normally enriched in neurons. Furthermore, the upregulation of microtubule-associated protein light chain 3, the degradation of p62/sequestosome 1, and the formation of autophagosomes occurred in treated cells, suggesting that TDCIPP exposure induces autophagy. However, pretreatment with the autophagy inhibitor 3-methyladenine suppressed TDCIPP-induced autophagy and reduced expression of the aforementioned cytoskeletal components. This correlated with a reduction in neurite outgrowth and numbers of neurite-bearing cells. Taken together, these results indicate that autophagy might promote TDCIPP-induced SH-SY5Y cell differentiation, which leads to an increase in expression of cytoskeletal components and neurite outgrowth. This study offers key insights into the mechanisms of neurotoxicity associated with this commonly used organophosphate.

1. Introduction

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is one of the most commonly used flame retardant additives in polyurethane foam. It is also used in baby products and residential furniture made after the 2005 phase-out of polybrominated diphenyl ethers (PBDEs) (Stapleton et al., 2011; Van der Veen and de Boer, 2012). Environmental monitoring studies showed that high concentrations of TDCIPP have been detected in indoor air (up to 40.1 μg/g in China; He et al., 2015), dust (up to 16.9 ng/m^3; Ren et al., 2016), and biota (freshwater fish: 89 ng/g lipid weight; Hallanger et al., 2015). In humans, TDCIPP and/or its metabolites have been detected in urine (including that of pregnant women; Butt et al., 2014), breast milk (Kim et al., 2014; Sundkvist et al., 2010), and placental tissue (Ding et al., 2016). Recently, studies reported that TDCIPP may have neurotoxic effects (Faust and August, 2011; Meeker et al., 2013; Ta et al., 2014; Li et al., 2017a,b). In particular, several in vitro studies have described neurotoxicity induced in cultured cells (Dishaw et al., 2011; Ta et al., 2014; Li et al., 2017a,b). Specifically, TDCIPP promotes the differentiation of PC12 cells into dopaminergic and cholinergic neurons, as indicated by the significant increase in expression of choline acetyltransferase and tyrosine hydroxylase, respectively (Dishaw et al., 2011). In undifferentiated PC12 cells, TDCIPP reduced DNA synthesis and overall cell numbers in a concentration-dependent fashion, and these effects were equivalent to or greater than a treatment with equimolar amounts of chlorpyrifos (Dishaw et al., 2011). Ta et al. (2014) reported that TDCIPP inhibited cell growth, increased apoptosis, altered cell morphology, changed gene and protein expression of regulatory proteins (CAMK2a, CAMK2β and GAP-43) and the structural proteins (tubullin-α, tubullin-β and NF-H), and ultimately led to cytotoxicity and neurotoxicity in PC12 cells (Ta et al., 2014). Recently, we showed that exposure to TDCIPP generates reactive oxygen species and induces apoptosis in SH-SY5Y cells by inducing

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ER stress and upregulating mitochondrial apoptotic pathways (Li et al., 2017a). Furthermore, we demonstrated that TDCIPP can also trigger autophagy, which protects against TDCIPP-induced apoptosis (Li et al., 2017b).

Neural differentiation, which is characterized by neurite outgrowth and other discrete morphological alterations, is controlled by the regulation of extrinsic signaling factors and intrinsic cell type-specific genes (Christie and Turnley, 2012). Neuronal differentiation is extremely complicated. It can occur in different cell types and is triggered by a variety of inducers (Tian et al., 2010; Agarwal et al., 2016). Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process of bulk degradation. It involves the sequestration of cytoplasmic components within a double-membraned structure, termed the autophagosome. The autophagosome is subsequently delivered to lysosomes for degradation (Levine, 2005). Accumulating evidence suggests a vital role for autophagy in the development and differentiation of the central nervous system (Boland and Nixon, 2006; Mizushima and Levine, 2010). Studies have shown that the induction of autophagy promotes differentiation in early neural stem cells and progenitors, mouse neuroblastoma cells (N2a cells), and human neuroblastoma cells (SH-SY5Y cells) (Plowey et al., 2008; Zeng and Zhou, 2008; Vázquez et al., 2012).

The cytoskeleton plays key roles in neurite outgrowth. Interactions between the highly dynamic cytoskeletal microtubules and microfilaments are essential for neurite growth and neuronal differentiation (Stiess et al., 2010). In the nervous system, the growth of neurites directly depends on the organization and function of microtubules and neurofilaments (Shea and Beermann, 1994). The dynamic properties of microtubules are modulated by microtubule-associated proteins (MAPs). MAP-2, a well-known member of the MAP family, is specific for neurons in the mammalian brain. MAP-2 interacts with microtubule filaments and plays an important role in the initiation of neurite growth (Mandelkow and Mandelkow, 1995). Recently, it was shown that the environmental neurotoxin tri-ortho-cresyl phosphate induces autophagy in differentiated SH-SY5Y cells, leading to degradation of cytoskeletal components and inhibition of neurite outgrowth (Chen et al., 2013). Phorbol myristate acetate (PMA) also triggers autophagy and promotes SH-SY5Y cell differentiation, as evidenced by the increase in expression of MAP-2 and β-TUBULIN (Zogovic et al., 2015). Cumulatively, these studies suggest that autophagy and cytoskeletal components play important roles in neuronal differentiation.

In this study, the SH-SY5Y cell line was selected as an in vitro model to investigate the effects of TDCIPP on cell differentiation. SH-SY5Y cells are undifferentiated, and can be induced to differentiate into neuronal-like cells in vitro by treatment with several agents, such as retinoic acid, tetramethylpyrazine, and PMA (Clagett-Dame et al., 2006; Cheung et al., 2009; Zogovic et al., 2015; Yan et al., 2015). Hence, this cell line is a classical in vitro model for neurobiological experiments, including studies of neuronal differentiation (Tonazzini et al., 2014). Here, we investigated cytotoxicity, cell differentiation, molecular markers of autophagy (microtubule-associated protein light chain 3 [LC3] and p62/sequestosome 1 [SQSTM1]), and the expression of various cytoskeletal components (NF-H, NF-L, and β-III-Tubullin) during TDCIPP-induced SH-SY5Y cell differentiation. This work sheds new light on the molecular and cellar mechanisms underpinning organophosphate neurotoxicity.

2. Materials and methods

2.1. Materials

TDCIPP (CAS No. 13674-87-8; >95.6% purity) was purchased from TCI Tokyo Chemical Industry Company (Tokyo, Japan). Primary antibodies against LC3, SQSTM1, and MAP-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Acridine orange (AO) and 4′-6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

The human neuroblastoma SH-SY5Y cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Culturing of SH-SY5Y cells was performed as previously described (Li et al., 2017a). Briefly, SH-SY5Y cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU penicillin, and 100 mg/ml streptomycin. Cultures were maintained in a humidified incubator with an atmosphere of 5% CO_2 and 95% air at 37 °C. The culture medium was replaced every 2 d and a sub-culture was generated once cells reached 80% confluency.

2.3. Cell viability assays

Cell viability was measured by using the MTT and LDH assays. In both assays, SH-SY5Y cells were seeded into a 96-well culture plate with 5×10^3 cells/well. Twenty-four hours later, cells were treated with different concentrations of TDCIPP (0, 1.25, 2.5, 5, and 10 μM) for 1, 3, or 5 d. Media containing TDCIPP was changed every 48 h. For the MTT assay, 20 μl MTT (5 mg/ml) was added to each well following TDCIPP incubation for 4 h at 37 °C. The media and excess MTT were then removed. An aliquot of 200 μl DMSO was added to dissolve the formazan crystals, and the absorbance was recorded at 570 nm using a microplate reader (Molecular Device, M2, Union City, CA). Cell viability was expressed as a percentage of the cell survival rate compared to the control.

For the LDH assay, LDH activity was measured using the LDH Detection Kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Briefly, following TDCIPP treatment, the supernatant was moved to a new 96-well plate. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and then lysed using 0.3% Triton X-100 dissolved in PBS for 30 min at 37 °C. LDH activity in the medium and in the cells was assayed according to the manufacturer's instructions. The absorbance of all samples was measured at 490 nm with a microplate reader (Molecular Device, M2, Union City, CA, USA). LDH release was expressed as a percentage of total LDH activity (LDH in the medium + LDH in the cells) according to the following equation: % LDH release = (LDH activity in the medium/total LDH activity) × 100%.

2.4. Quantification of neurite-bearing cells and neurite outgrowths

Cells were seeded into 6-well plates (2×10^4 cells/well) in DMEM/F12 supplemented with 10% FBS for 24 h. For control cells, the medium was replaced with DMEM/F12 supplemented with 3% FBS. The treatment group was incubated with 2.5 μM TDCIPP for 5 d, during which the medium was exchanged every 48 h. Neurite outgrowth was visualized by inverted phase contrast microscopy at 0, 3, and 5 d. The percentage of cells with neurites was determined by quantifying images using the Image J open source software. Only cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated (Dehmelt and Halpain, 2004). Images were taken from at least four random fields per sample. At least three samples were evaluated per experimental group. The length of the longest neurite was measured in at least 100 cells in randomly chosen fields.