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The involvement of ROS overproduction and mitochondrial dysfunction in PBDE-47-induced apoptosis on Jurkat cells

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ABSTRACT

2,2′,4,4′-Tetrabromodiphenyl ether (PBDE-47), as one of the congeners of polybrominated diphenyl ethers (PBDEs), is widely present and threatens the human health in many aspects. This study aims to investigate the toxic effects of PBDE-47 on cell viability, apoptosis, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) of Jurkat cells *in vitro*. The results showed that PBDE-47 significantly inhibited the viability of Jurkat cells in a dose-dependent manner by alamar blue assay. Significant induction of apoptosis was detected in Jurkat cells at 25–100 μM by propidium iodide staining, accompanied with overproduction of ROS and downregulation of MMP. Furthermore, N-acetyl-L-cysteine (NAC), a widely used ROS scavenger, significantly reduced the PBDE-47-induced apoptosis by decreasing ROS level and mediating recovery of the MMP. In conclusion, the results of this study suggest that PBDE-47 could induce apoptosis in Jurkat cells and ROS and mitochondrial dysfunction play important roles in the apoptotic process.

Introduction

The polybrominated diphenyl ethers (PBDEs) are a group of halogenated organic brominated flame retardants (BFRs) and are widely used in industrial products. PBDEs have the no-chemical bonding characteristics and can be easily released to the environment during product use and disposal (Vonderheide et al., 2008). Therefore, PBDEs have been commonly found in the air, soil, sediment, water of natural environments and media around human living circumstances such as buildings, furniture and sewage (Hazrati and Harrad, 2006; Costa et al., 2008). PBDEs potentially contain 209 different congeners, varying in both number and position of bromination. 2,2′,4,4′-Tetrabromodiphenyl ether (PBDE-47) is one of the most prevalent congeners in both abiotic and biotic environments (de Wit, 2002).

PBDEs can cause serious health problems. Previous studies have shown that PBDEs can cause hepatic toxicity and disrupt thyroid hormone status (Hu et al., 2007; Richardson et al., 2008; Tseng et al., 2008). Some evidences have shown that PBDEs are also developmental neurotoxicants. PBDEs can cause spontaneous behavior dysfunction, learning and memory defects and disrupt habituation capability during developing stage in neonatal mouse (Branchi et al., 2002, 2003; Viberg et al., 2007). Exposure to PBDE-47 at 18 and 36 mg/kg body weight can also induce the immunotoxicity by decreasing the quantity of splenocytes in adult mice (Darnerud and Thuvander, 1999). A commercial mixture of PBDEs named DE-71 can also increase the percentage of neutrophils and significantly decrease the percentage of lymphocytes in mink (Martin et al., 2007). Furthermore, DE-71 can induce the formation of reactive oxygen species (ROS) on human neutrophil granulocytes, potentially through NADPH oxidase activation (Reistad and Mariussen, 2005). These data suggest a close relationship between ROS formation and immunotoxicity induced by PBDEs. However, the toxic effect and mechanism of PBDEs on immunocytes *in vitro* are still poorly understood.

Jurkat cells are an immortalized line of T lymphocyte cells, frequently employed as a T-cell lymphoma model system to study toxicity of environmental contaminants (Walsh et al., 2008; Yao et al., 2007). In the present study, Jurkat cells were chosen to explore the cytotoxicity of PBDE-47 and its underlying mechanism, with a focus on apoptosis induction and the role of ROS and mitochondrial dysfunction in apoptotic process.

Materials and methods

Reagents

PBDE-47 (purity > 99%) was purchased from Chem Service Inc (West Chester, USA); Alamar blue assay kit from Shanghai Sunbio Medical Biotechnology Co., Ltd. (Shanghai, China); Propidium iodide (PI) and RNAse A from Sigma (St Louis, MO, USA);

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Rhodamine 123 (Rh123) from Beyotime Biotechnology Inc. (Nantong, China); 2, 7-Dichlorofluorescin-diacetate (DCFH-DA), N-acetyl-L-cysteine (NAC) and Dimethyl sulfoxide (DMSO) from Amresco (purity > 99.9%) (USA). All reagents used in the experiment were of analysis grade or higher purity.

Cell culture and treatment

Jurkat cells (population doubling time 20.7 ± 2.2 h) were obtained from Cell Bank of Chinese Academic of Science (Shanghai, PR China) and maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, USA) and 100 U/ml penicillin/streptomycin in a humidified incubator at 37 °C and 5% CO_2.

Jurkat cells were exposed to different concentrations of PBDE-47 by adding an appropriate volume of the stock solution in DMSO to the culture medium. PBDE-47 was dissolved in DMSO, and the final culture concentration of DMSO in all experiments was 0.5% (v/v) or less. Control cells were cultured in medium containing 0.5% DMSO. For cell viability assay, Jurkat cells were treated with 5, 10, 25, 50 and 100 μM PBDE-47 for 24, 48 and 72 h ($n=6$). Based on the results from viability assay, the 48 h time point was chosen to perform the apoptosis analysis with the doses 5, 10, 25, 50 and 100 μM PBDE-47 ($n=6$). To evaluate the ROS and mitochondrial membrane potential (MMP), Jurkat cells were exposed to 5, 10, 25, 50 and 100 μM PBDE-47 for 24, 48 and 72 h ($n=6$). The effects of NAC at 1 and 5 mM were evaluated by co-treatment with 50 μM PBDE-47 for 48 h.

Cell viability assay

The cytotoxic effect of PBDE-47 on Jurkat cells was analyzed by the alamar blue assay (Hallman et al., 2000). Briefly, Jurkat cells were plated on a 96-well (100 μl/well) microplate at a density of 5×10^5 cells/ml. PBDE-47 or NAC at various concentrations was added and the plate was incubated at 37 °C in humidified 5% CO_2 for 24, 48 and 72 h. 10 μl solution containing alamar blue was added to each well and incubated for an additional 4 h. The fluorescence density was measured with a fluorescence spectrophotometer using 540 nm Ex and 590 nm Em filter settings.

Measurement of apoptosis

The proportion of apoptotic cells was measured with PI staining of DNA fragmentation by flow cytometry (Tavakkol-Afshari et al., 2008). Briefly, Jurkat cells were seeded into cell culture flask at a density of 5×10^5 cells/ml. Treated cells were harvested and washed twice with PBS, fixed in ice-cold 70% ethanol and stored at 4 °C overnight. Prior to analysis, the cells were washed twice again with PBS, suspended in 1 ml PBS containing 100 μg/ml RNase A and 50 μg/ml PI. The cells were then incubated at 4 °C for 30 min in the dark. Flow cytometric analyses were carried out using a FACScan flow cytometer (Becton, USA) with 488 nm Ex and 630 nm Em filter settings. Cell Quest software was used to determine apoptosis.

Detection of reactive oxygen species (ROS) level

The intracellular ROS level was determined using DCFH-DA by the method of Myhre et al. (Myhre et al., 2003), with some modifications. Briefly, cells were harvested and suspended in PBS. The cells were then treated with 10 μM DCFH-DA and incubated in dark for 30 min at 37 °C. The fluorescence was detected with a fluorescence spectrophotometer using 485 nm Ex and 525 nm Em filter settings.

Determination of the mitochondrial membrane potential (MMP)

Rh123, which can bind specifically to mitochondria, has been used in numerous investigations to estimate MMP (Xiang et al., 2006) with some modifications. Briefly, treated cells were harvested and washed, then incubated with Rh123 (5 μg/ml) in PBS for 60 min in the dark at 37 °C. The fluorescence was measured with a fluorescence spectrophotometer using 507 nm Ex and 529 nm Em filter settings.

Statistical analysis

Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($P<0.05$ or $P<0.01$) between the means of control and treated cells were analyzed by Dunnett's test. Data were expressed as means ± SD.

Results

PBDE-47 affected the cell viability of Jurkat cells

The viability of Jurkat cells was significantly inhibited by PBDE-47 in each treated group ($P<0.05$) (Fig. 1). The greatest inhibition happened 72 h after 100 μM treatment, with a loss of viability to 54% (Fig. 1A). The loss of cell viability in PBDE-47-treated groups was presented in a dose-dependent manner ($R=-0.9372$ for 72 h, -0.8696 for 48 h, -0.8899 for 24 h with $P<0.01$) (Fig. 1B).

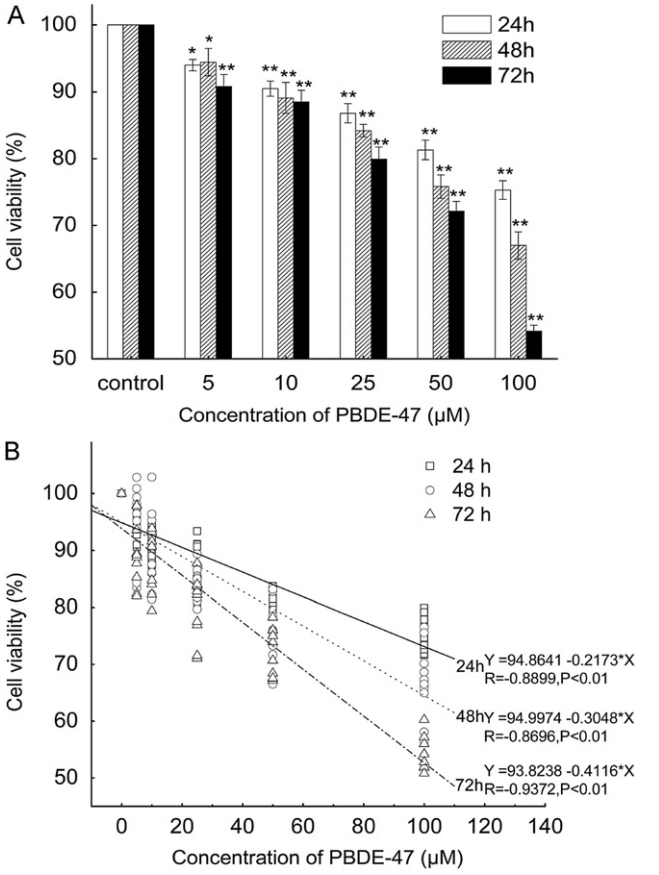

Fig. 1. Effects of PBDE-47 on the viability of Jurkat cells. (A) Jurkat cell viability; (B) linear regression fitting model of the viability loss ($n=6$, $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle control).

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PBDE-47-induced apoptosis in Jurkat cells

PBDE-47 significantly induced apoptosis in Jurkat cells at 25, 50 and 100 μM. The apoptotic rates at 48 h after treatment were 9.33%, 14.29% and 23.46%, respectively ($P<0.05$) (Fig. 2).

PBDE-47 caused ROS overproduction in Jurkat cells

Significant increase in ROS level was detected in each treated group except at 48 h after 5 μM PBDE-47 treatment ($P<0.05$). Particularly, ROS level was much higher than that in control group at 100 μM for 72 h with a 4.4-fold increase (Fig. 3).

PBDE-47-induced downregulation of MMP in Jurkat cells

As shown in Fig. 4, Jurkat cells treated with PBDE-47 exhibited the decrease in MMP significantly except at 5 μM (24 and 48 h) and 10 μM (24 h) ($P<0.05$). In particular, the MMP dropped to 54% of that in control group 48 h after 100 μM PBDE-47 treatment, and decreased further to 32% at 72 h (Fig. 4).

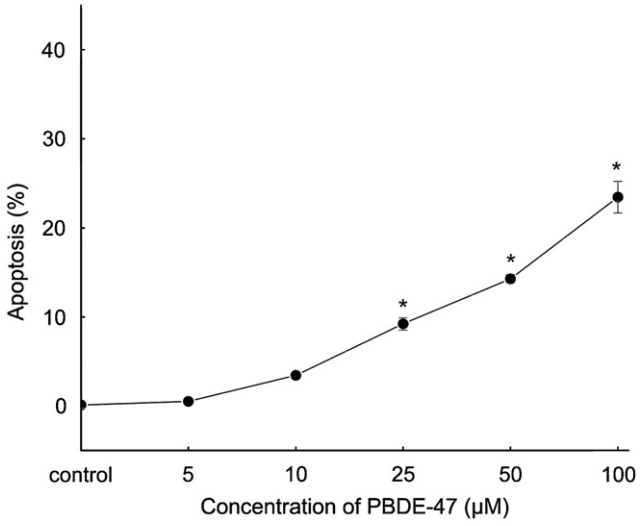

Fig. 2. Percentage of apoptosis induced by PBDE-47 in Jurkat cells for 48 h ($n=6$, $^{*}P<0.05$ compared with vehicle control).

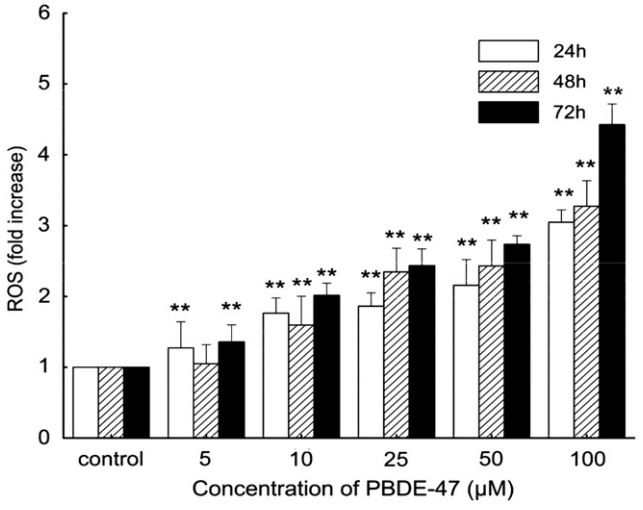

Fig. 3. ROS levels affected by PBDE-47 in Jurkat cells ($n=6$, $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle control).

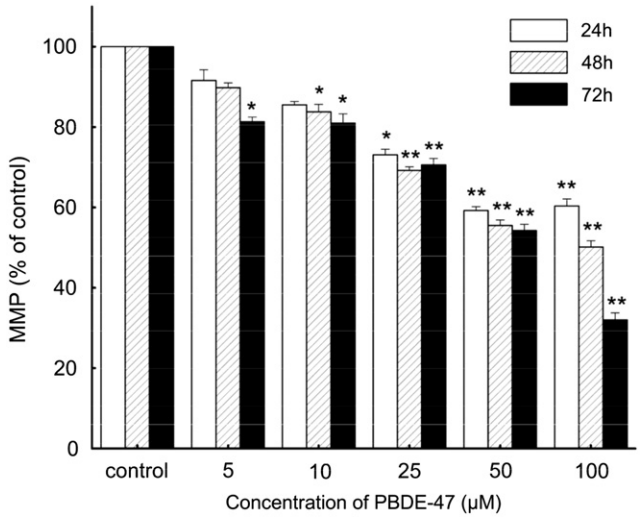

Fig. 4. MMP affected by PBDE-47 in Jurkat cells ($n=6$, $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle control).

The protective effects of NAC on PBDE-47-induced cytotoxicity

NAC was used to explore the mechanism of cytotoxicity induced by PBDE-47 at 48 h time point. The results showed that NAC could reduce the apoptotic rate from 14.29% to 6.74% and 2.89% at 1 and 5 mM, respectively ($P<0.05$) (Fig. 5A). These data are significantly different from that in control group ($P<0.05$). Moreover, NAC significantly reduced the intracellular ROS level and reversed the MMP of Jurkat cells ($P<0.05$) (Fig. 5B).

Discussion

Studies on PBDEs including PBDE-47 have shown that these compounds can cause cytotoxicity in several normal and tumor cell lines of liver organ and nervous system *in vitro* (He et al., 2008; Hu et al., 2007; Madia et al., 2004; Shao et al., 2008a). Consistently, our results indicated that PBDE-47 significantly decreased the viability of Jurkat cells in a dose-dependent manner. The cytotoxicity observed in our experiment was slightly different from that in other cell lines. For example, the study in neuroblastoma cells have shown that the cell viability is less than 50% of the control when cells are exposed to 10 μg/ml PBDE-47 (He et al., 2008). While in the present study, our data showed that the viability was 54% at the maximal PBDE-47 dose (100 μM) and longest exposure time (72 h). The results indicated that PBDE-47 caused less cytotoxicity in Jurkat cells than in other cell lines.

As is known, apoptosis is the process of programmed cell death that may occur in multicellular organisms, especially when exposed to environmental toxicant. We observed that apoptosis in Jurkat cells occurred after exposure to 25–100 μM PBDE-47. These changes were accompanied by the viability decrease in Jurkat cells. Taken together, these results implied that apoptosis might be the main reason for the reduction in cell viability. Considering the current results and the facts that some PBDEs including PBDE-47 can induce apoptosis in other cell lines (Hu et al., 2007; Madia et al., 2004), we suggest PBDEs may induce a similar apoptotic response in different cell lines. Our results indicated PBDE-47-induced immunotoxicity *in vitro* through apoptosis and provided a probable explanation for the decrease in immunocytes as observed in the *in vivo* experiments (Martin et al., 2007). It is worthwhile to note that in the current experiment PBDE-47 induced apoptosis at relatively high concentrations that are of less relevance to exposures *in vivo*.

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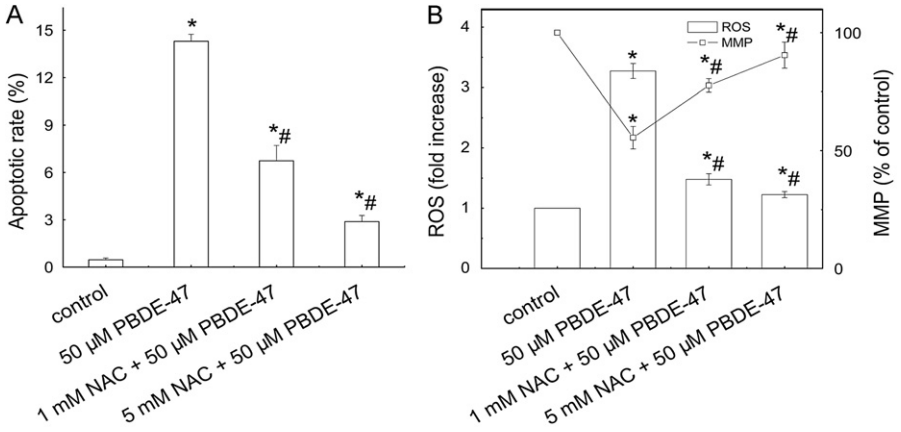

Fig. 5. (A) The effects of NAC on PBDE-47-caused apoptosis in Jurkat cells ($n=6$) at 48 h time point; (B) NAC reduced the intracellular ROS level and reversed the MMP in Jurkat cells at 48 h time point ($n=6$, $^{*}P<0.05$ compared with vehicle control; $^{\#}P<0.05$ compared with 50 μM PBDE-47).

However, many factors including length of exposure, constituents of the exposure solution and total media volume can influence the accumulation of PBDE-47 in cells (Mundy et al., 2004), which suggests further studies are needed to investigate the physiological relevance of the toxicity observed *in vitro*.

In order to study the mechanism of PBDE-47-induced apoptosis, we investigated the effects of PBDE-47 on the ROS formation and MMP. In the present study, the significant overproduction of ROS was detected in PBDE-47-caused cytotoxicity in a dose-dependent manner at all time points. At 25–100 μM, ROS production increased as the exposure time extended. Similar results are obtained in human neuroblastoma cells and human fetal liver hematopoietic stem cells (He et al., 2008; Shao et al., 2008b). Moreover, we also found MMP decreased significantly, concomitant with an increase in intracellular ROS. As ROS have the potential to induce the collapse of MMP, consequently trigger a series of mitochondria-associated events including apoptosis (Armstrong, 2006; Kim et al., 2005), our findings accordingly suggested the involvement of ROS and mitochondrial dysfunction in PBDE-47-induced apoptosis.

NAC is used as an ROS scavenger to study the role of ROS in the cell apoptotic process (Wang et al., 2009), which can protect cells against oxidative damage in two ways at a certain concentration, by reacting with H_2O_2 extracellularly as a direct antioxidant and increasing the cytoplasmic reserve of glutathione (Han et al., 2007; Sadowska et al., 2007). In the present research, we found that NAC had a suppressive effect on PBDE-47-induced apoptosis. Furthermore, our results showed that the presence of NAC blocked the intracellular ROS generation and MMP collapse. Inhibitory effect of NAC on intracellular ROS increase induced by PBDE-209 in HepG2 cells is also observed (Hu et al., 2007). Changes in mitochondrial permeability during apoptosis are the important events which lead to the collapse of the mitochondrial transmembrane electrochemical gradient. Subsequently cytochrome c and other proapoptotic molecules are released into the cytoplasm, activating apoptosis (Fleury et al., 2002). Being the major sources of ROS production, mitochondria could be the main targets of ROS attack. Therefore, co-treatment with NAC can protect the mitochondrial from oxidative injury and attenuate the apoptosis by decreasing the ROS level in Jurkat cells. Our results also suggest that PBDE-47-induced ROS overproduction may cause the collapse of the MMP, initiating the apoptotic process.

In conclusion, PBDE-47 could significantly induce apoptosis in Jurkat cells, indicating immunotoxicity happened *in vitro*. The experimental results on ROS and MMP, together with the protective effect of NAC, suggest that ROS overproduction and mitochondrial dysfunction may play key roles in the apoptotic process induced by PBDE-47.

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