Journal of Hazardous Materials 185 (2011) 24–28

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

PCB congeners induced mitochondrial dysfunction in Vero cells

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ARTICLE INFO

Article history:
Received 6 May 2010
Received in revised form 6 August 2010
Accepted 12 August 2010
Available online 21 August 2010

Keywords:
PCBs
Vero cells
Mitochondria
Apoptosis

ABSTRACT

Two PCB congeners were assessed for their cytotoxicity on Vero cells, in the attempt to compare their structure-activity relationship and to investigate the role of mitochondria involved in toxicity. Flow cytometry was used to monitor the changes of mitochondrial membrane potential ($\Delta\psi_m$), cell size and apoptosis rate. Treatments of Vero cell cultures with both PCB 126 and PCB 153 resulted in loss of cell viability in our experimental conditions. In *ortho*-substituted PCB 153 treated cells, loss of cell viability was accompanied by decreased $\Delta\psi_m$ and cell shrinkage. The coplanar congener, PCB 126, had no significant effects on $\Delta\psi_m$ or cell size in this time period of exposure. These studies showed that PCB 153 is more toxic than coplanar PCB 126 to Vero cells within 24 h exposure. The cytotoxicity mechanism caused by coplanar or non-coplanar PCB congener was different, and apoptosis might be the main cell death pathway in PCB 153 treated cells.

1. Introduction

Polychlorinated biphenyls (PCBs) are a structurally related group of environmental contaminants that represent significant environmental and human health concerns. Their high lipophilicity has resulted in bioaccumulation in various organisms through the food chain, and many PCB congeners have been detected in human blood, milk, and other tissues [1,2]. PCBs consist of up to 209 different congeners, and the biological effects and toxicity of individual congener greatly depend on its structure [3,4]. Congeners with one or no chlorine substituent in the *ortho* positions may assume a planar configuration, with a pattern of toxicity similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Congeners with several chlorine substitutions in the *ortho* positions are frequently referred to be a non-coplanar configuration and thus elicit different toxicity from coplanar ones [5]. Most mechanistic studies about the toxicity of PCBs have been focused on coplanar, dioxin-like congeners. However, it is becoming increasingly clear that certain *ortho*-substituted, non-planar PCB congeners also exhibit important biological activities, and play important role in cytotoxicity [6].

Mitochondria are multitasking organelle surrounded by a double membrane configuration. As one of the most important organelles, mitochondria are involved in a number of cellular functions [7]. They are not only the major energy source in cell, but also the main site of cellular metabolism [8]. Moreover, mitochondria play a central role in complex physiological processes including cell proliferation, differentiation and apoptosis [9,10]. Shaping of mitochondria could have impacts on mitochondrial function and cell metabolism.

PCB exposure has been involved in renal toxicity from both *in vitro* and *in vivo* experiments [11–13]. Treatments with PCB mixtures were reported to cause the loss of cell viability and accelerate apoptosis in renal cell cultures [14]. The dysfunction of mitochondria has been described as the damage caused by PCB exposure [15,16]. Since previous studies have implicated mitochondria in PCB-induced cytotoxicity, we chose Vero cells from African green monkey kidney as the test model to search the actions of the two representative PCB congeners on cell viability and mitochondria functions. The objectives of this study were: (1) to assess the potential of cytotoxicity caused by testing PCB congeners; (2) to compare the contribution of dioxin-like PCB 126 and *ortho*-substituted PCB 153 on cell death; (3) to identify the actual cell death pathway caused by different PCB congeners; and (4) to characterize the molecular mechanisms involved in the PCB-induced cytotoxicity, particularly the role of mitochondria.

2. Materials and methods

2.1. Chemicals and reagents

PCBs 126 and 153 were purchased from Accustandard, Inc. (New Haven, CT, USA). PI/RNase staining buffer was purchased from BD Pharmingen, Inc. (San Diego, CM, USA). Rhodamine 123 was pur-

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doi:10.1016/j.jhazmat.2010.08.061

chased from Sigma, Inc. (St. Louis, MO, USA). PCBs were dissolved in DMSO such that the final DMSO concentration was never greater than 0.2% (v/v).

2.2. Cell culture

Vero cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin (100 IU ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. MTT cytotoxicity assay

The effect of PCBs on cell proliferation was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. MTT test provides a quantitative measure of surviving and/or proliferating cells by determining the amount of formazan crystals produced by metabolic activity in treated versus untreated control cells. Briefly, exponentially growing Vero cells were trypsinized and plated at a density of 1×10^4 per well in 96-well plates overnight to allow attachment. The cell cultures were treated with different PCB congeners for 24 h. Following incubation, cells were incubated with 5 $mg\,ml^{-1}$ MTT for 4 h at 37 °C. The medium was then aspirated and DMSO was used to dissolve the crystals. Absorbance was measured at 570 nm on SpectraMax Plus 384 microplate reader.

2.4. Detection of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was monitored by loading Vero cells with 5 $\mu g\,ml^{-1}$ rhodamine 123. After 30 min incubation at 37 °C in the dark, the cells were centrifuged for 5 min at 2000 rpm, washed, re-suspended and transferred to flow cytometry tubes (1×10^6 cells ml^{-1}). Data from at least 15,000 cells were analyzed with CELLQuest software.

2.5. Apoptosis detection

To quantify apoptotic cells, PCB treated Vero cells were monitored by flow cytometry following staining with propidium iodide (PI) [17–19]. Briefly, Vero cells were seeded into 6-well culture plates at a density of 1×10^5 cells per well. The cells were treated with 10 $\mu g\,ml^{-1}$ PCB congeners for 24 h. After culturing, the cells were digested with trypsin and washed with PBS. Then they were fixed with ice-cold 70% ethanol overnight, washed with PBS, and treated with 500 μl of PI/RNase staining buffer for 30 min at 37 °C. The cells were analyzed on a FACSCalibur, and CELLQuest software was used for data acquisition. A minimum of 15,000 events were collected per sample. The data were analyzed using ModFit LT software.

2.6. Morphological alteration of mitochondria

Following 24 h PCB exposure, Vero cells were harvested, fixed in 4% (v/v) glutaraldehyde, and post-fixed in 1% (v/v) osmium tetroxide. After dehydration through a series of graded alcohol concentrations and acetone, the samples were rinsed and impregnated with Spurr's resin. The ultra-thin sections were prepared and mounted on copper grids for viewing in the JEM-1230 transmission electron microscope.

2.7. Statistical analysis

Data were expressed as means ± S.D. and analyzed by analysis of variance (ANOVA) using SPSS software version 16.0. A p value of less than 0.05 was considered to be significant.

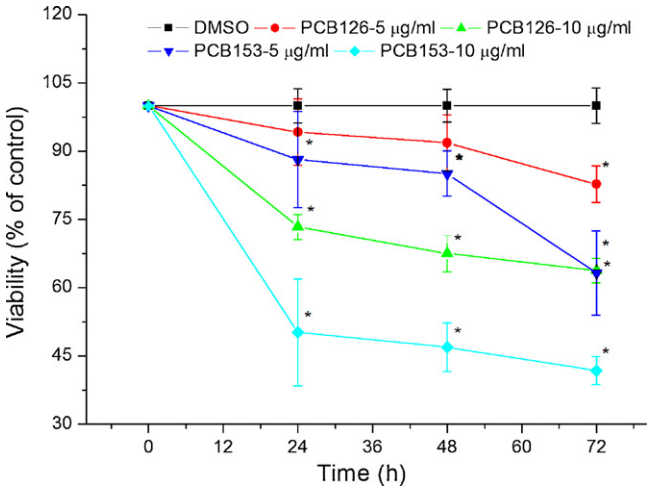

Fig. 1. Effects of different PCB congeners on Vero cell viability. The cells were incubated with 10 $\mu g\,ml^{-1}$ coplanar PCB 126 and *ortho*-substituted PCB 153 for 24 h. * Significant different from control ($p < 0.05$ from two-way ANOVA). All data show mean values ± S.D.

3. Results

3.1. Cytotoxicity assays

Fig. 1 shows the effects of PCB 126 and 153 on cell viability measured after 24, 48 and 72 h exposure. Results indicated that both the coplanar PCB 126 and *ortho*-substituted PCB 153 caused loss of cell viability. Furthermore, the non-coplanar PCB 153 was a more efficient inducer of Vero cell death over the exposure period.

3.2. Effects of PCBs on mitochondrial membrane potential

In our experiment, several indications of PCB caused disruption of mitochondrial function were found. Fig. 2 shows the effects of exposure to two PCB congeners on rhodamine 123 fluorescence polarization in Vero cells. After 24 h incubation with PCB 153, the fluorescence polarization declined by almost 50%, whereas a similar concentration and duration exposure to PCB 126 had a different but not significant effect.

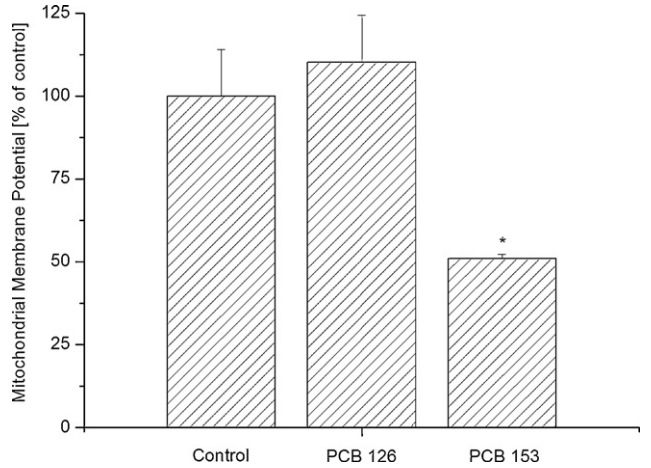

Fig. 2. Measurement of mitochondrial membrane potential in Vero cells induced by PCB congeners (10 $\mu g\,ml^{-1}$). * Indicates $p < 0.05$ compared to DMSO control. All results shown are mean ± S.D.

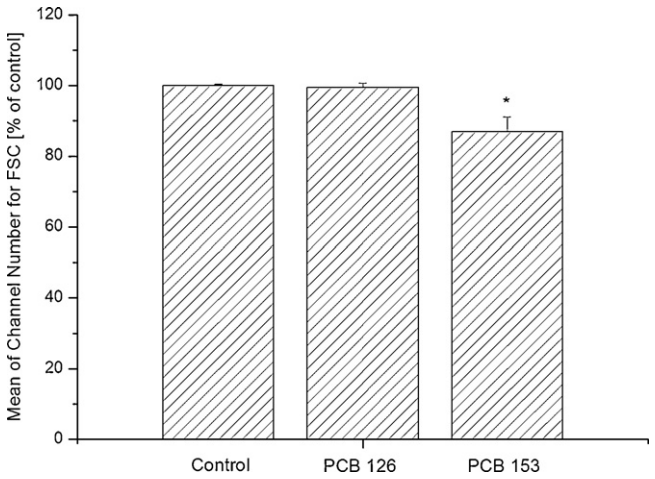

Fig. 3. Effects of different PCB congeners on Vero cell size, represented by the mean channel number of forward scatter (FSC). Forward scatter reflects cell size, and therefore a decrease in forward scatter indicated shrinkage of average cell size. All data show mean values ± S.D. * Indicates $p < 0.05$ compared to DMSO control. The reduction in forward scatter indicates shrinkage of the cells.

3.3. Effects of PCBs on cell size

Significant cell swelling would be a reason to cause cell death [20]. To test whether PCBs induced cell death via this mechanism, we monitored cell size upon exposure to both PCB congeners. Coplanar PCB 126 did not alter cell size, but the toxic *ortho*-substituted PCB 153 caused a decrease in cell size (Fig. 3). These observations indicated that cell swelling was not the mechanism of PCB-induced cell death.

3.4. Effects of PCBs on apoptosis

Apoptotic cells could be separated from normal ones by their lower DNA contents, shown as a sub-G1 cell population in cell cycle [21–24]. Flow cytometric analysis of DNA content showed that PCB 153 induced cellular apoptosis effectively (Fig. 4). PCB 126 did not significantly change the percentage of apoptotic cells. The apoptotic rates induced by PCB 126 and PCB 153 were 1.50% and 19.21%, respectively. PCB 153 was a more effective apoptosis inducer compared with PCB 126.

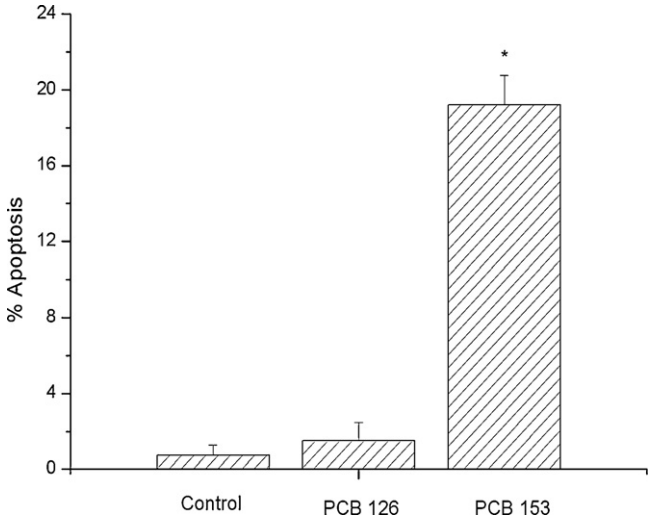

Fig. 4. Cell apoptosis rate after 24 h exposure to 10 μg ml^{-1} PCB congeners. * Significantly different from control ($p < 0.05$ from two-way ANOVA). All results shown are mean ± S.D.

3.5. Effects of PCBs on morphological alteration

In control cells, mitochondrial images were abundant and appeared round or elongated, depending on the plane of sectioning; these organelle had a characteristic structure with transversal cristae (Fig. 5A). After PCB 126 treatment, the tumefaction of mitochondria was obvious (Fig. 5B). In PCB 153 treatment group, the cytoplasm appeared as a mottled, lumpy and diffuse mass distributed throughout the cytosol (Fig. 5C).

4. Discussion

Studies in our laboratory and others indicated that treatments with PCB congeners led to cytotoxicity and caused cell death in cell cultures [25,26]. Apoptosis and necrosis were suggested to be two main mechanisms in cell death. Necrosis is usually accompanied by cell swelling and apoptosis by cell shrinkage [27]. Mitochondria have been associated with cell death caused by PCB exposure. There are several reports that PCBs may influence mitochondrial function [28–30], therefore, mitochondria injury might be a candidate mechanism of cell death. To identify the role of mitochondria in PCB-induced cytotoxicity, we have combined biochemical and morphological techniques to establish the relevance of mitochondria in the death of Vero cells induced by PCBs.

Cytotoxicity caused by PCB exposure was detected by MTT test, which is based on the conversion of the dye by mitochondrial succinate dehydrogenase. MTT results are usually interpreted as indicative for the mitochondrial reductive activity [31]. In the concentration utilized, both PCB 126 and PCB 153 resulted in the loss of cell viability in our experimental conditions. Our results indicated the mitochondrial damage caused by PCB exposure.

The morphological alteration of mitochondria caused by PCB exposure has been reported [32,33], however, the physiological consequences of this alteration are not known. Since *ortho*-substituted PCBs can alter mitochondria by inhibition of the electron transport chain [28,34], one predicted effect of non-coplanar PCBs would be the impairment of mitochondrial function. To test this hypothesis, we used rhodamine 123 to detect mitochondrial membrane potential ($\Delta\psi_m$). *Ortho*-substituted PCB 153, but not dioxin-like PCB 126, caused a dramatic drop in $\Delta\psi_m$. Meanwhile, this $\Delta\psi_m$ drop was accompanied by the reduction of cell viability. $\Delta\psi_m$ might be one important factor for the cytotoxicity induced by *ortho*-substituted PCB 153. However, in spite of a significant decrement of cell viability, PCB 126 caused no decline but a slight increase of $\Delta\psi_m$. The $\Delta\psi_m$ alone cannot be the major factor for the cytotoxicity caused by PCB 126 exposure. In the concentration utilized, the cell death pathway originated by the treatment of Vero cell cultures from PCB 126 or PCB 153 was different.

As one of the most important organelle, mitochondria play a central role in the cellular metabolism [35,36] and signal transduction [37–39]. It has been proved that mitochondria was implicated in a variety of key events of apoptosis such as disruption of energy metabolism; cytochrome *c* release and caspase activation [40]. The collapse of $\Delta\psi_m$ is one of the earliest events in apoptosis [41]. Based on the reduction of $\Delta\psi_m$ observed in PCB 153 treated cells, apoptosis might be the cell death mechanism caused by *ortho*-substituted PCB 153 exposure. To test whether non-planar PCB 153 induces cell death via apoptosis, we monitored cell size upon exposure to two PCB congeners. Coplanar PCB 126 did not alter cell size, but the *ortho*-substituted PCB 153 caused a decrease in cell size. These observations indicated that cell swelling was not the mechanism of PCB-induced cell death. Cell shrinkage is characteristic of apoptotic cell death, not necrotic. Our present data showed the decline $\Delta\psi_m$ as well as the cell shrinkage, which indicate that cell death mechanism caused by *ortho*-substituted PCB 153 exposure might

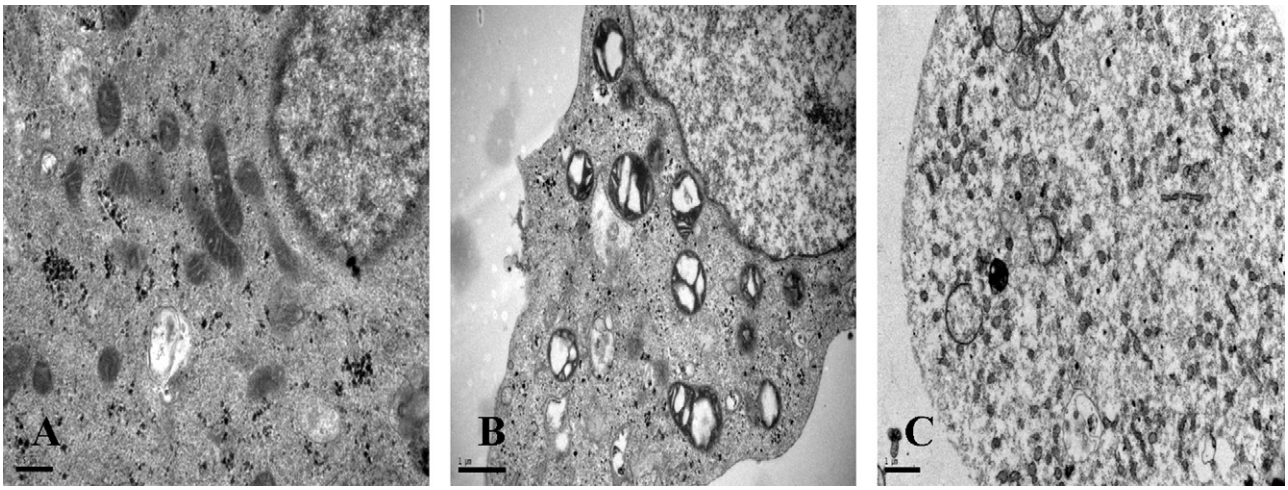

Fig. 5. Electron micrographs (TEM) of mitochondria alterations induced by PCBs. Cells were incubated in presence of (A) DMSO, (B) 10 μg ml^{-1} PCB 126 and (C) 10 μg ml^{-1} PCB 153 for 24 h.

be apoptosis. Flow cytometric results further provided powerful proof that the cell death pathway induced by the *ortho*-substituted PCB 153 was apoptosis.

PCB congeners bind to different cellular target sites and cause adverse effects by a variety of different mechanisms, depending on their three-dimensional structure and their substitution pattern [29]. The different cytotoxic effects between PCB 126 and PCB 153 may be associated with their own chemical structure. The chlorine atoms are relatively bulky; the presence of chlorines in the *ortho* positions causes the PCB molecule to take a three-dimensional structure, whereas with the chlorines in the *meta* and *para* positions, the molecule assumes a planar configuration. The three-dimensional structures of the *ortho*-substituted congeners cause sufficient perturbation of the membrane lipids and thus give rise to changes in physiological functions.

5. Conclusions

The present study suggested that treatment of Vero cell cultures with both PCB 126 and PCB 153 resulted in the loss of cell viability in our experimental conditions. *Ortho*-substituted PCB 153 induced a greater loss of cell viability than coplanar PCB 126, preceded by mitochondrial structural and functional damage, reduction in $\Delta\psi_m$ and cell size. Mitochondria play an important role in *ortho*-substituted PCB 153 induced cell death, and apoptosis would be one important cell death mechanism in PCB 153 exposure.

Acknowledgements

This work was supported by the Fundamental Research Funds for the Central Universities, and the Program for Changjiang Scholars and Innovative Research Team in University (IRT0536). We are grateful to thank Dr. Naveedulla from Zhejiang University for the modification of English expression.

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