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Oligomeric proanthocyanidins alleviate hexabromocyclododecane-induced cytotoxicity in HepG2 cells through regulation on ROS formation and mitochondrial pathway

An Jing [a,*], Chen Cen [a], Wang Xiu [a], Zhong Yufang [a], Zhang Xinyu [a], Yu Yingxin [a], Yu Zhiqiang [b]

[a] Institute of Environmental Pollution and Health, School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, PR China
[b] State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, PR China

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ABSTRACT

Hexabromocyclododecane (HBCD), a type of brominated flame retardants (BFR), has become ubiquitous organic contaminants in recent years. However, studies on HBCD toxicity and the related molecular mechanisms are so far limited. The objective of the present study was to investigate the effects of oligomeric proanthocyanidins (OPCs) on cytotoxicity induced by HBCD and the underlying molecular mechanisms. HepG2 cells were treated with HBCD and/or OPCs at different concentrations, and cell viability, cell apoptosis, reactive oxygen species (ROS) production, cellular Ca^{2+} level, mitochondrial membrane potential ($\Delta\Psi$), cytochrome C (Cyt-c) release, and nuclear factor-erythroid 2-related factor 2 (Nrf2) proteins expression were evaluated. Results showed that HBCD induced toxic effects in HepG2 cells in a concentration-dependent manner. HBCD at high concentrations (40 and 60 μM) caused a significant decrease of cell viability and led to elevated cell apoptosis ratio, intracellular Ca^{2+} level, cytoplasmic Cyt-c level, and ROS production, together with a loss of $\Delta\Psi$ and mobilization of Nrf2. Pretreatment with OPCs effectively attenuated the cytotoxic effects and ROS production, as well as mitochondrial responses induced by HBCD. Thus, OPCs could alleviate cytotoxicity in HepG2 cells induced by HBCD through regulation on intracellular Ca^{2+} level and ROS formation in a mitochondrial pathway.

1. Introduction

Hexabromocyclododecane (HBCD) is widely used as additive brominated flame retardants (BFR) in polystyrene foams, building materials, upholstery textiles, and electrical equipments (BSEF, 2012; de Wit, 2002; Zhang et al., 2008a,b). With the increase of production in the world, concentrations of HBCD in environment media have been rising in recent years. HBCD has become a class of ubiquitous organic contaminants recently and is under consideration to be included in persistent organic pollutants (POPs) (Birnbaum and Bergman, 2010).

As a highly lipophilic compound, HBCD has a tendency to be accumulated in the fatty tissues of organisms, and starvation could increase the redistribution of HBCD into liver and brain. Animal experiments indicated that liver is a major target organ for HBCD exposure in mammals (Ema et al., 2008; Gebbink et al., 2008; Tada et al., 2006). A long-term exposure to HBCD could result in pathological changes in liver of mammals, such as hepatic enlargement, liver nodules, fatty infiltration, liver necrosis, and even liver tumors (Palace et al., 2008; Saegusa et al., 2009; van der Ven et al., 2006). A sublethal dose (5 μM) of HBCD caused hepatotoxicity in zebrafish, which was demonstrated through cellular stress, metabolism disruption, and apoptosis of liver cells (Kling and Forlin, 2009).

Some toxicological studies have shown that oxidative stress is an important mechanism in HBCD-mediated cytotoxic effects. Zhang et al. (2008a,b)investigated the cytotoxicity of HBCD enantiomers in HepG2 cells and found that production of ROS was positively correlated with lactate dehydrogenase (LDH) release. Deng et al. (2009) also reported that HBCD could induce significant oxidative stress and apoptosis through caspase pathway in zebrafish embryos. Thus, we reasoned that antioxidants might protect cells against oxidative stress and cytotoxic effects induced by HBCD.

Oligomeric proanthocyanidins (OPCs) are a class of novel potent natural antioxidants, exhibiting multiple biological activities through scavenging free radicals. Praphasawat et al. investigated the anti-mutagenic and anti-oxidative properties of OPCs in TK6 cells and found that OPCs treatment significantly reduced the micronucleus frequency caused by mitomycin C and DNA damages induced by H_2O_2 (Praphasawat et al., 2011). The effect of OPCs on UV-induced melanogenesis showed that OPCs had photoprotection potential in human melanocytes by scavenging intracellular ROS, improving cell viability, adjusting cell cycle, and inhibiting TRP1 and TRP2 protein expression of melanogenic enzymes (Zi et al., 2009). Moreover, OPCs play an important role in alleviating inflammatory reactions caused by diabetic oxidative stress through

* Corresponding author. Tel./fax: +86 021 66137736.
E-mail address: peace74839@shu.edu.cn (J. An).

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suppression of ROS generation and elevation of the glutathione/oxidized glutathione (GSH/GSSG) ratio (Yokozawa et al., 2012). In addition to the capability to scavenge ROS, OPCs could also stimulate nitric oxide (NO) production and activate protein kinase B/nitric oxide synthase (Akt-NOS) signaling to protect cardiomyocytes from ischemia and reperfusion injury (Shao et al., 2009).

In the present study, we focused on the human hepatoma cell line HepG2 as a model of in vitro tests, since HepG2 cells are widely used in toxicological studies of chemical and environmental risk assessments (Vidic et al., 2009; Baderna et al., 2011). Moreover, the expression of antioxidant and xenobiotic metabolizing enzymes, often induced or inhibited by various chemical substances are similar in HepG2 cells and primary human hepatocytes (Wilkening et al., 2003). The protective effects of OPCs against cytotoxicity induced by HBCD and the potential molecular mechanisms in HepG2 cell line were investigated.

2. Materials and methods

2.1. Materials

Commercial product Grape Seed Extract purchased from Winherb Medical Science Co., Ltd. (Shanghai, China) is standard herbal extracts from *Vitis vinifera* linne with a purity of 98% OPCs as tested by UV. HBCD was purchased from TCI (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM, high glucose concentration), D-Hank's, and fetal calf serum (FBS) were obtained from GIBCO (Invitrogen Corp., Paisley, UK). Cell counting kit-8 (CCK-8), Fluo 4-AM and pluronic F-127 (F-127) were purchased from Dojindo (Kumamoto, Japan). Nuclear and cytoplasmic extraction reagent kit (NE-PER) and bicinchoninic acid (BCA) protein assay were purchased from Thermo Fisher Scientific Inc (Waltham, USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) detection kit was purchased from Mbchem (Shanghai, China). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), tert-butyl hydroperoxide (tBHP), rhodamine 123 (Rh123), and dimethyl sulfoxide (DMSO) were purchased from Sigma (Saint Louis, USA). Nitrocellulose membrane was purchased from Millipore (Darmstadt, Germany).

All antibodies were purchased from following vendors: anti-Cyt-c (Epitomics, Burlingame, USA), anti-Nrf2 (Epitomics, Burlingame, USA), anti-GAPDH (Multisciences Biotechnology, Hangzhou, China), anti-rabbit IgG (H + L)/HRP and anti-mouse IgG (H + L)/HRP (Dingguo, Beijing, China). Other reagents were from Sigma (Saint Louis, USA) and were analytical grade chemicals, if not stated otherwise. All experiments were carried out at least in triplicate.

2.2. Cell culture and treatments

The human hepatocellular carcinoma cell line HepG2 was cultured in DMEM supplemented with 10% FBS, 0.33% sodium bicarbonate, and antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin) at 37 °C in a 95:5 air/CO_2 water-saturated atmosphere. For all experiments, cells were seeded at 5×10^4 cells/mL, passaged every 2 days and used for experiments in exponentially growing phase. HepG2 cells were treated with different concentrations of HBCD (0, 10, 20, 40, 60 μM) dissolved in DMSO and/or OPCs (0, 10, 20, 40 μg/mL) dissolved in phosphate-buffered saline (PBS). Controls contained vehicle alone with a final concentration of DMSO or PBS at 0.1% v/v. After treatments, cells were washed twice with PBS and harvested with lysis buffer.

2.3. Cell viability

HepG2 cells were treated with different concentrations of HBCD (0, 10, 20, 40, 60 μM) for different time (1, 2, or 3 days) and then cell viability was measured with CCK-8 assay. Suitable concentrations (40 and 60 μM) with obvious reduced cell viability were selected to evaluate the effect of OPCs on HBCD induced cytotoxicity. For HBCD/OPCs-combined treatments, cells were seeded in 96-well plates for 24 h at a concentration of 2500 cells per well and were starved (deprivation of serum) for 24 h to achieve a synchronization effect and to exclude possible influence of cell cycle on proliferation. Then, cells were treated with different concentrations of (0, 10, 20, 40 μg/mL) OPCs for 6 h and then exposed to HBCD (40 or 60 μM) for 2 days. Cell viability was measured by adding 10 μL of CCK-8 reagent in 90 μL DMEM per well to cell culture media and then incubating at 37 °C for 1 h. Absorbance was then measured on a plate reader (Biorad, iMark, Hercules, USA) at 450 nm.

2.4. Assessment of apoptosis

Annexin V-FITC/PI double staining assay was used to measure cell apoptosis ratio. At first, HepG2 cells were treated with different concentrations of HBCD (0, 10, 20, 40, 60 μM) for 1, 2, or 3 days. Then 40 and 60 μM were selected to evaluate the effect of OPCs on HBCD induced apoptosis. HepG2 cells were pretreated with OPCs at different concentrations (0, 10, 20, 40 μg/mL) for 6 h and then exposed to HBCD (40 or 60 μM) for 2 days. After treatments, cells were harvested with 0.25% trypsin and washed twice with PBS. For each sample, approximately 10^6 cells were suspended in 1 × binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/mL. Then cells were incubated with Annexin V-FITC and PI at 37 °C for 30 min in the dark. The stained cells were immediately analyzed by flow cytometry using a FL2 detector (Becton Dickinson, San Jose, USA) with the excitation wavelength at 488 nm. Data were analyzed with Cell Quest Software (Becton Dickinson, San Jose, USA).

2.5. Measurement of reactive oxygen species (ROS)

The level of intracellular ROS was assessed using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (An et al., 2012). To evaluate the effects of OPCs on HBCD-induced ROS generation, HBCD/OPCs-treated cells in 35-mm petri dishes were washed twice with D-Hanks and then were incubated with 10 μM DCFH-DA at 37 °C for 30 min in the dark. After washing with D-Hanks three times again, cells were suspended in D-Hanks. The cell suspensions were observed under a fluorescence microscope (Olympus BX-51, Tokyo, Japan) and fluorescence intensity was estimated by Image-pro plus 6.0 software.

2.6. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was measured with Rh123 assay. Cells cultured in 35-mm petri dishes were treated with different concentrations of HBCD (0, 10, 20, 40, 60 μM) for 1, 2, or 3 days. In the combined-treated groups, HepG2 cells were pretreated with OPCs at different concentrations (0, 10, 20, 40 μg/mL) for 6 h and then exposed to HBCD (40 or 60 μM) for 2 days. After washing with D-Hank's, cells were incubated with 10 μM of Rh123 in culture media at 37 °C for 30 min, washed three times with D-Hank's, and observed under the fluorescence microscope. Fluorescence intensity was evaluated by Image-pro plus 6.0 software.

2.7. Measurement of cellular Ca^{2+} level

Calcium measurement was performed as described elsewhere with some modifications (An et al., 2012). HepG2 cells were seeded on small slides in 35-mm petri dishes for specified time. Then cells