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Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling

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ABSTRACT

Nrf2/ARE pathway plays an important role in adapt to oxidative stress caused by pro-oxidants and electrophiles through up-regulating phase II detoxifying enzymes. Our previous study has demonstrated that PCB quinone exposure causes severe cellular oxidative stress (Toxicology *In Vitro* 26 (2012) 841–848). There are no reports describing the ability of PCB quinone on Nrf2/ARE activation. In the present study, we found that exposure to PCB29-pQ resulted in a significant increase in Nrf2 and Keap1 expression in total protein, as well as the Nrf2 targeting genes, including NQO1 and HO-1. Next, immunocytochemistry analysis identified the accumulation of Nrf2 in nucleus subsequent to PCB29-pQ treatment. The increased Nrf2 and constant Keap1 expression in nucleus suggested the dissociation of Nrf2/Keap1 complex. Similarly, mRNA level of Nrf2 was elevated significantly with PCB29-pQ treatment, but not Keap1. Additionally, PCB29-pQ treatment led to significant up-regulation of the mRNA level of antioxidant enzymes, NQO1 and HO-1, in a concentration-dependent manner. Electrophoretic mobility shift assay and luciferase reporter assay further confirmed the formation of Nrf2–ARE complex. PCB29-pQ treatment has no effect on mitogen-activated protein kinase signaling, however, phospho-AKT was up-regulated and GSK-3β was down-regulated. Pretreatment with LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), suppressed the phosphorylation of AKT and inhibited PCB29-pQ induced Nrf2/HO-1 activation, meanwhile, GSK-3β expression was increased accordingly. At last, reactive oxygen species (ROS) scavengers inhibited PCB29-pQ induced Nrf2 activation partly. These results suggested that Nrf2 activation by PCB29-pQ in HepG2 cells is associated with ROS and AKT pathway but not MAPK signaling, the activation of Nrf2/ARE may be an adaptive response to oxidative stress.

1. Introduction

Polychlorinated biphenyls (PCBs) are synthetic chlorinated aromatic compounds, consisting of 209 possible congeners. They have been used extensively in the electricity generating industry as dielectrics in capacitors and flame retardants in cooling fluids and lubricants [1]. PCBs were produced from the 1930's through the 1980's with an estimated total production of 1.5 million tons [2] and now distributed worldwide. Due to their lipophilic nature, they accumulate easily in the lipid bilayer and fat deposits of the body and biomagnifies in the food chain [3]. Potential health consequences of PCB exposure include immunotoxicity, neurotoxicity, endocrine toxicity and *etc.* [4]. Several studies suggested that an important underlying mechanism of PCB-mediated toxicity is due to the induction of cellular oxidative stress [5–7].

Recently, much attention has been paid to the toxicological activity of the metabolites of PCB, including hydroxylated and quinone-type metabolites [8,9]. Hydroxylated PCBs (OH-PCBs) are most abundant metabolites which have been found in human blood with a total percentage ranges from 13% to 44% to total PCBs [10–12]. Hydroxylated PCBs are readily metabolized further to quinone-type metabolites, which mediated ROS can cause cellular damage through alkylation reactions with DNA, proteins and lipids [9]. We have recently shown that PCB quinones cause severe cellular oxidative stress [13]. Moreover, addition of antioxidant has

Abbreviations: ARE, antioxidant response element; Cul3, Cullin 3; DAPI, 4′,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; EpRE, electrophile-responsive element; ERK, extracellular signal-regulated protein kinase; GSH, glutathione; GSK-3β, glycogen synthase kinase 3 beta; HO-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; LA, lipoic acid; MAPKs, mitogen-activated protein kinases; MEK, MAPK kinase; NAC, *N*-acetylcysteine; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PCB, polychlorinated biphenyl; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; VE, vitamin E.

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been shown to ameliorate oxidative damage induced by PCB quinones, which authenticated the role of ROS and oxidative stress in PCB quinones cytotoxicity.

To counteract oxidative stress, cells have complicated mechanisms of defense against this toxicity, one of the most important mechanism involves the activation of Keap1/Nrf2/ARE pathway [14], which leads to the expression of cytoprotective enzymes, such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) and *etc*. The coordinated transcriptional response is mediated through the antioxidant response element (ARE) or electrophile-responsive element (EpRE) located at the enhancer regions of these genes. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a member of the cap 'n' collar family basic region-leucine zipper transcription factor. Nrf2 is the central transcriptional regulator in ARE-driven gene expression in response to oxidative stress. Previous study demonstrated that H_2O_2-treatment induce endogenous Nrf2 protein in rat cardiomyocytes [15]. Nrf2 knockout mice exhibit a severe deficiency in the coordinated gene regulatory program and extreme susceptibility to oxidative damage indicated the importance of Nrf2 in antioxidant defense [16]. Therefore, Nrf2-mediated antioxidant response plays a vital role in maintaining intracellular redox homeostasis [17].

Since it is well understood that Nrf2 is a key regulator in cellular adaptive response to oxidative stress [18] and PCBs induce oxidative damage *in vitro* and *in vivo* [19,20]. However, there has no conclusive answer to whether Nrf2 is involved in the oxidative stress response induced by PCBs or their metabolites. Our recent finding demonstrated that PCB quinone type derivative, 2,3,5-trichloro-6-phenyl-[1,4]benzoquinone (PCB29-pQ), causes severe cellular oxidative stress in HepG2 cells [13]. In the present study, we investigated the effects of PCB29-pQ on Nrf2-mediated antioxidant response in HepG2 cells.

2. Materials and methods

2.1. Materials

The 2,3,5-trichloro-6-phenyl-[1,4]benzoquinone (PCB29-pQ, Fig. 1) were synthesized and characterized as previously described [21], maintained in −20 °C freezer under argon atmosphere. The purity of test compound was confirmed by HPLC analysis and a satisfied purity was greater than 98%. A stock solution of PCB quinone (50 mM) was prepared in DMSO before use. RPMI1640 medium was obtained from Gibco Co. (Gibco BRL, Grand Island, NY, USA), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), LY294002 and pARE-luciferase vector were purchased from Beyotime Institute of Biotechnology (Haimen, China). Keap1, NQO1, HO-1, β-actin polyclonal antibodies and nuclear/cytosol fractionation kit were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). AKT, phospho-AKT, ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK and GSK-3β polyclonal antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China), Nrf2 monoclonal antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Secondary antibody Alexa Fluor® 594 goat anti-rabbit IgG was purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). RT-PCR amplification primers of Nrf2, Keap1, NQO1, HO-1 and GAPDH were synthesized by Dingguo Biotechnology Co., Ltd. (Beijing, China). Lipofectamine 2000 transfection reagent, PRL-SV40 vector and dual-luciferase reporter assay system kit were supplied by Promega, USA. All other chemicals used were of the highest commercial grade.

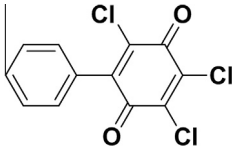

Fig. 1. Chemical structure of PCB29-pQ.

2.2. Cell culture and treatment

The human hepatoma cell line HepG2 was purchased from Third Military Medical University, Chongqing, China. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (HyClone, USA) and antibiotics (100 U/mL penicillin, 100 lg/mL streptomycin) at 37 °C and 5% CO_2. Normally, HepG2 cells were seeded in 6-well culture plates and permitted to adhere at 37 °C for 24 h, then incubated with PCB29-pQ for a further 6 h in serum-free medium. PCB29-pQ stock solution was added directly to cell culture media to attain a final concentration of 25 or 50 μM, control cells were treated with equal amount of DMSO only. DMSO was always <0.1% in the culture. LY294002 inhibition assay was performed by pre-incubated of LY294002 for 30 min, then treated cells with PCB29-pQ for 6 h. ROS scavengers assay was performed by pre-incubated of antioxidants for 1 h then treated cells with PCB29-pQ for 6 h.

2.3. Immunocytochemistry

Immunocytochemistry analysis was carried out according to the method described by Hwang et al. [22]. Cells grown on poly-*L*-lysine-coated coverslips were treated with PCB29-pQ for a indicated time, then, cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton-X and blocked in 10% non-fat dry milk in blocking buffer at 4 °C for 12 h. Primary anti-Nrf2 polyclonal antibody was added (1:200 dilution) and cells were incubated for 2 h. Then, cells were incubated with secondary antibody, Alexa Fluor® 594 goat anti-rabbit IgG (1:150 dilution) for an additional 1 h. After two further washes in PBS, the sections were counterstained with 1 μg/mL of DNA dye 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Finally, cells were analyzed on a fluorescence microscope (OLYMPUS IX71).

2.4. Cytosolic and nuclear protein extraction

Cell lysates were added to cytosolic extraction buffer containing protease inhibitors and a reducing agent (20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate and 1 mM Na_3VO_4), vortexed vigorously for 15 s. After incubation for 10 min on ice, cell lysates were centrifuged at 12,000 rpm for 10 min and the cytoplasmic fraction supernatants were transferred to new tubes and stored at −20 °C until used. Nuclear proteins were fractioned using a nuclear/cytosol fractionation kit according to the manufacturer's instructions. Pellets were re-suspended in nuclear extraction buffer, after centrifugation at 15,000 rpm for 10 min, and the supernatant was kept as the nuclear extract and stored at −80 °C until used.

2.5. Western blot analysis

Equal amounts of protein (100 μg per sample) were separated by electrophoresis in 10% or 12.5% SDS–PAGE and transferred to nitrocellulose membrane. After blocking with 10% skimmed milk, proteins were, respectively incubated with rabbit polyclonal antibodies of Nrf2, Keap1, HO-1, NQO1, ERK, phospho-ERK, JNK, phospho-JNK, p38, phospho-p38, AKT, phospho-AKT, GSK-3β, Lamin B and β-actin at room temperature for 4 h. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature. Finally, immunoreactive