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Short Communication

PCB-77 disturbs iron homeostasis through regulating hepcidin gene expression

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ABSTRACT

PCBs are a family of persistent environmental toxicants with a wide spectrum of toxic features, such as immunotoxicity, hepatoxicity, endocrine disruption effects, and oncogenic effects. To date, little has been done to investigate the potential influence of PCB exposure on iron metabolism. Deregulated iron would lead to either iron deficiency or iron excess, coupled with various diseases such as anemia or hemochromatosis. Iron metabolism is strictly governed by the hepcidin–ferroportin axis, and hepcidin is the key regulator that is secreted by hepatocytes. Here, we found that PCB-77 could go through plasma membrane and accumulate in hepatocytes. PCB-77 was demonstrated to suppress hepcidin expression in HepG2 and L-02 hepatocytes. Moreover, hepatic hepcidin was observed to be inhibited in mice upon administration of PCB-77. Due to reduced hepcidin concentration, serum iron content was increased, with a significant reduction of splenic iron content. Together, we deciphered the molecular mechanism responsible for PCB-conducted disturbance on iron homeostasis, i.e. through misregulating hepatic hepcidin expression.

1. Introduction

Polychlorinated biphenyls (PCBs) with 209 congeners, according to the position of the chlorine atoms in the biphenyl ring, are a class of lipophilic inert compounds. PCBs, originally manufactured commercially for industrial applications, were appreciated for their insulating and flame resistant properties (Safe, 1994); however, commercial productions were banned in the United States in 1979. PCBs, with slow rates of biotransformation in our biosphere, cause a variety of adverse effects on human health including reproductive, neurological and endocrine disorders (Loch-Caruso, 2002; Portigal et al., 2002; Schantz et al., 2003). Although a number of studies have investigated the toxicities of PCBs from various aspects, such as reproductive toxicity (Richthoff et al., 2003), inhibition of growth (COLE and PLAPP, 1974), immunotoxicity (Smialowicz et al., 1989; Tryphonas, 1995), hepatoxicity (Yoshimura et al., 1979), endocrine disruption effects (Birnbaum, 1994), neurotoxicity (Kodavanti and Tilson, 1996), enzyme induction (Burgin et al., 2001), and oncogenic effects (Lucena et al., 2001; Wolff et al., 2000; Zheng et al., 2000), the molecular mechanisms underlying these adverse effects are still elusive. Moreover, the potential influence of PCBs on iron metabolism has not been studied thus far.

Iron is an essential constituent of the internal environment and an absolute element for nearly all forms of life. Iron participates in multiple biological processes, e.g. DNA synthesis (Ganeshaguru et al., 1980) and protein formation (Duce et al., 2010). It is crucial to maintain iron homeostasis; otherwise both iron overload and iron deficiency result in toxicities through promoting oxidative stress (Knutson et al., 2000). A tight control of the balance for iron intake, utilization and storage is therefore fundamentally important for cells to keep cells healthy. Hepcidin, a 25-amino-acid peptide secreted by the liver, is the central regulator of systemic iron homeostasis (Elizabeta Nemeth et al., 2004). It binds to ferroportin, the major iron exporter in mammals, and causes internalization of ferroportin, leading to iron retention inside cells (Ganz and Nemeth, 2011). Hepcidin primarily inhibits intestinal iron absorption by blocking ferroportin-conducted iron transportation from the gastrointestinal system to the circulating system (Evstatiev and Gasche, 2012). It also prevents iron release from ferroportin-expressing cells, such as macrophages and hepatocytes, resulting in iron accumulation in local tissues (Bekri et al., 2006). Hepcidin expression is fundamentally governed by the BMP family members (Andriopoulos et al., 2009) and the IL-6 family cytokines (Banzet et al., 2012) through erythropoietic demand, iron burden and inflammatory stimuli (Ganz, 2010). Hepcidin deficiency is associated with increased serum iron and leads to a few iron-related disorders, such as nervous system diseases (Jeong and David, 2003), hepatocirrhosis (Fei et al., 2006) and even cancers (Bystrom et al., 2012; Nemeth and Ganz, 2006).

The aim of this study was to evaluate the potential effects of PCBs on iron homeostasis. Here, we found that PCB-77, a representative PCB congener that is widely spread in the environment, could

Abbreviations: BMP, bone morphogenetic protein; DMSO, dimethylsulfoxide; ERE, estrogen response elements; GC–MS, gas chromatography–mass spectrometry; IL-6, interleukin-6; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCB-77, 3,3′,4,4′-tetrachlorobiphenyl; PCBs, polychlorinated biphenyls; qRT-PCR, quantitative real-time polymerase chain reaction.

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significantly alter systemic iron homeostasis through misregulating hepatic hepcidin.

2. Materials and methods

2.1. Chemicals and reagents

PCB-77 (3,3′,4,4′-tetrachlorobiphenyl) was purchased from the AccuStandard Inc. Dimethyl sulfoxide (DMSO) (Solarbio Inc., China) was used to dissolve PCB-77. The final concentration of DMSO in culture medium was <0.1%.

2.2. Cell culture

Human hepatic carcinoma cell line HepG2 and hepatic epithelial cell line L-02 were obtained from the Shanghai Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 100 U/mL penicillin–streptomycin (Hyclone) at 37 °C under 5% CO_2.

2.3. Cytotoxicity assays

Cytotoxicity was assessed by the MTT assay following the instructions from the manufacturer (Roche). Briefly, cells were serum starved for 12 h and were then inoculated into 96-well plates at a concentration of 6.0×10^3 cells/well upon different treatments. Cells were cultured for another 24 h, and 20 μL MTT (5 mg/mL) was added into each well followed by incubation for 4 h. Thereafter, 200 μL DMSO was added into each well, and 96-well plates were read at 490 nm on a microplate reader (Thermo) after shaking. Lactate dehydrogenase (LDH) release into culture media after exposure to PCB-77 was detected with a kit of CytoTox-ONETM Homogeneous Membrane Integrity Assay according to the manufactures' instructions (Promega).

2.4. Gas chromatography–mass spectrometry (GC–MS)

Quantification of PCB-77 was performed using an Agilent 6890 GC, coupled with 5973 MSD. The MS analyses were conducted in an electron capture negative ionization (ECNI) mode, and separation was performed using a fused silica capillary column (RTX-1614, 30 m, 0.25 mm ID and 0.1 μm film thickness). The carrier gas used was helium, with a flow rate of 1.0 mL min^{-1}, and methane was used as the reaction gas. The GC oven temperature was programmed as follows. The initial temperature was 120 °C maintained for 1 min, and was then increased to 150 °C at a rate of 30 °C min^{-1}, followed by a 2.5 °C min^{-1} ascent to 280 °C. The temperatures for the injector, the transfer line and the ion source were 290 °C, 280 °C, and 230 °C, respectively.

2.5. Animal experiments

All animal care and surgical procedures were approved by the Animal Ethics Committee at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Female Balb/c mice of six weeks old were purchased from the Vital River Laboratories, China, and housed under a sterile and pathogen-free environment. Mice in the treatment group received a single dose of intraperitoneal (i.p.) injection of PCB-77 diluted in corn oil (80 mg/kg). The control mice received coil only. The iron content in diet was 480 mg/kg in the current study.

2.6. RNA extraction and qRT-PCR analysis

Total RNAs were isolated from cells using Trizol according to the manufacturer's instructions (Invitrogen). Tissue samples were first pulverized in liquid nitrogen and then total RNAs were extracted with TriPure following the instruction provided by the manufacturer (Roche). Quantitative measurements of gene expression were carried out with SYBR Green qPCR master mix (Qiagen) on qPCR Systems Mx3005P (Stratagene). Primer sequences for PCR reactions were as follows, mouse hepcidin: forward, 5′-CTGAGCAGCACCACCTATCTC-3′, and reverse, 5′-TGGCTCTAGGCTATGTTTTGC-3′; mouse HPRT: forward, 5′-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3′, and reverse, 5′-CCCTGAAGT ACTCATTATAGTCAAGGGCAT-3′; human hepcidin: forward, 5′-CCTGACC AGTGGCTCTGTTT-3′, and reverse, 5′-CACATCCCACACTTTGATCG-3′; human GAPDH: forward, 5′-GAAGGTGAAGGTCGGAGT-3′, and reverse, 5′-GAAGATGGTGATGGGATTTC-3′. HPRT and GAPDH were used as an internal control for human or mouse samples, respectively.

2.7. Determination of iron content

Serum iron content was determined with a kit following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Tissue non-heme iron was assayed following a standard protocol as previously described (Liu et al., 2007).

2.8. Statistical analysis

Independent *t* test or one-way ANOVA test was used to analyze experimental data. Data were shown in mean ± SE. $P < 0.05$ were considered statistically significant.

3. Results and discussion

Liver is the preferential organ for PCB accumulation due to their significant characteristic of lipophilicity (Kodavanti et al., 1998). Meanwhile, liver is also the primary organ in governing iron homeostasis, and hepatocytes are the predominant cell type for hepcidin synthesis which is the central hormone in regulating iron uptake, distribution and storage (Drakesmith and Prentice, 2012; Ganz, 2010). To accurately uncover the potential effects of PCBs on iron metabolism, we first attempted to choose sublethal concentrations of PCB-77 in cell treatment by avoiding significant toxicity to hepatocytes. As shown in Fig. 1A, the MTT assay revealed that there was no significant cytotoxicity of PCB-77 to HepG2 cells within the concentration range from 0.01 μM to 2 μM. Membrane integrity could be assessed by the release of a cytosolic enzyme, LDH, into culture medium (Hawkins and Abrahamse, 2006). We thus evaluated the loss of membrane integrity of HepG2 cells upon treatment with PCB-77. The LDH release assay indicted that PCB-77 at the current concentrations had no effect on the extracellular LDH concentrations (Fig. 1B), in agreement with the results from the MTT assay (Fig. 1A).

The biological effects of environmental pollutants closely rely on their bioavailability by cells (Serrano et al., 2009). Bioavailability is the fraction that reaches into cells of the total amount administered. In the present study, we measured the bioavailability of PCB-77 by HepG2 cells. Based on the viability assays as shown in Fig. 1, a nontoxic concentration of 0.1 μM was chosen to assess the uptake of PCB-77 by HepG2 cells. The uptake of PCB-77 was analyzed with the approach of GC–MS. PCB-77 treatment in culture medium at 0.1 μM (equivalent to 59.6 ng for all cells in the culture well) for 24 h resulted in accumulation of 2.26 ng PCB-77 in 1.0×10^6 HepG2 cells (Fig. 1C). Therefore, the amount of PCB-77 that entered 1.0×10^6 cells accounted for 3.8% of the total amount in the culture medium (Fig. 1C). These results demonstrated that PCB-77 could readily enter cells without damaging cells.

We thereafter assay the possible influence of PCB-77 treatment on hepcidin expression. As shown in Fig. 2A, the mRNA level of hepcidin in HepG2 cells was significantly inhibited by more than 50% at 0.1 μM for 24 h ($P = 0.001$). To substantiate this finding, we examined hepcidin expression in another hepatocyte cell line L-02. Hepcidin expression was significantly repressed by 59% in L-02 cells with PCB = 77 treatment at 0.1 μM for 24 h (Fig. 2B, $P < 0.05$), similar to the finding in HepG2 cells. Dysregulation of hepcidin production results in a variety