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, hepatotoxicity, 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.

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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-Carusio, 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), hepatotoxicity (Yoshimura et al., 1979), endocrine disruption effects (Birnbbaum, 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% CO₂.

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 μ L MTT (5 mg/mL) was added into each well followed by incubation for 4 h. Thereafter, 200 μ L 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 μ m 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 oil 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'-CTGAGCAGACCACCTATCTC-3', and reverse, 5'-TGGCTCTAGGCTATGTTTTGC-3'; mouse HPRT: forward, 5'-GCTTGCTGTTGAAAAGGACCTCTCGAAG-3', and reverse, 5'-CCCTGAAGTACTATTATAGTCAAGGGCAT-3'; human hepcidin: forward, 5'-CCTGACCAGTGGCTCTGTTT-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 $\pm$ 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 μ M to 2 μ M. 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 indicated 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 μ M 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 μ M (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 μ M 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 μ M for 24 h (Fig. 2B, *P* < 0.05), similar to the finding in HepG2 cells. Dysregulation of hepcidin production results in a variety

Fig. 1. Cytotoxicity and bioavailability of PCB-77 in HepG2 cells. (A) The cell viability assay through the MTT assay. HepG2 cells were treated with PCB-77 at various concentrations as indicated for 24 h, followed by the MTT assay ($n = 6$). (B) LDH release assay. Membrane integrity was determined by the release of LDH into culture medium from HepG2 cells after PCB-77 treatment at concentrations as indicated for 24 h ($n = 6$). (C) Uptake of PCB-77 by HepG2 cells. Cells were exposed to PCB-77 at 0.1 μ M for 24 h, and were then collected for PCB-77 quantification with GC-MS ($n = 6$).

of iron disorders. For example, hepcidin deficiency leads to the development of systemic iron overload due to excessive iron absorption and iron release from macrophages, as hepcidin knock-out mice displayed severe iron overload (Nicolas et al., 2001). Hepcidin deficiency is coupled to a few iron diseases, such as hemochromatosis and β -thalassemia (Pietrangelo, 2011).

To confirm the inhibitory effect of PCB-77 on hepcidin expression, we looked into hepcidin expression in mice upon PCB-77 administration. We performed animal experiments with short-term administration for 6 h, 12 h, 24 h, 2 days and long-term administration for 10 days. Mice were i.p. injected with PCB-77 and were then sacrificed after 6 h, 12 h, 24 h, 2 days or 10 days. Regarding the short-term administration of PCB-77, the qRT-PCR results showed that hepatic hepcidin expression was consistently suppressed more than 65% at 6 h, 12 h and 24 h after PCB-77 administration, compared to that in the mice without PCB administration (Fig. 3A, $P < 0.05$). There was no significant alteration to the body iron status in the PCB-77 administrated mice, as the concentrations of serum iron, hepatic iron and splenic iron were not significantly changed with a slight increase in serum iron and a slight decrease in splenic iron at 24 h (Fig. 3B–D, $P > 0.05$), suggesting a slow adjustment in serum and spleen iron in response to hepcidin changes. However, there were significant alterations in the body iron status 2 days after PCB-77 administration in mice. The hepcidin level was reduced by 45%

2 days after PCB-77 administration, compared to that in the control mice (Fig. 4A, $P < 0.05$). Subject to the reduction of hepatic hepcidin for 2 days, serum iron was elevated by more than 2.3 fold in mice treated with PCB-77, compared to the untreated mice (Fig. 4B, $P < 0.05$). Meanwhile, the splenic iron was decreased by approximately 40% in mice administrated with PCB-77, compared to the control mice (Fig. 4C, $P < 0.05$). The liver iron concentration kept consistent with or without PCB-77 administration (Fig. 4D, $P > 0.05$).

With respect to the long-term administration of PCB-77 for 10 days, the hepcidin expression was consistently repressed by approximately 65%, and the serum iron concentration was increased by 60%, compared to the control mice (Figs. 4E and F, $P < 0.05$). However, spleen iron content was similar between the PCB-77-treated mice and the control mice for 10 days (Fig. 4G, $P > 0.05$). Liver iron content did not significantly change (Fig. 4H, $P > 0.05$), similar to that in mice upon acute PCB-77 treatment (Figs. 3–4). The repletion of iron in spleen after chronic PCB-77 treatment is presumably due to consecutive high-level iron absorption from small intestine subject to continuous hepcidin attenuation, leading to sufficient iron supply for various organs including spleen. Additionally, phagocytosis of senescent erythrocytes by macrophages in spleen could partially replenish lapsed iron during the long-term exposure.

Iron is an essential element necessary for numerous biological processes. For example, iron-containing proteins exert a variety of functions including gas transport, electron transfer in the respiratory chain, catalysts of biodegeneration or biosynthesis, transcription factors and repressors, enzymes of intermediate metabolism, and DNA synthesis and repair (Evstatiev and Gasche, 2012). However, deregulated iron homeostasis is involved in the development of diverse diseases including cancers (Bystrom et al., 2012) and neurodegenerative brain disorders (Benarroch, 2009). Liver-derived hormone, hepcidin, centrally modulates iron homeostasis by inducing the internalization and degradation of ferroportin, the only known cellular iron exporter that is expressed by duodenal enterocytes, macrophages and hepatocytes (Elizabeta Nemeth et al., 2004). Liver is also physiologically responsible for the uptake, storage, and disposal of nutrients, drugs and toxins including environmental pollutants. Previous studies have demonstrated that hepatic hepcidin expression is essentially regulated by erythropoiesis demand (Pak et al., 2006) and inflammation (Nemeth et al., 2004). Thus far, no study has been preformed to investigate the potential regulation of hepcidin expression by environmental pollutants. PCBs, as a

Fig. 2. Inhibition of hepcidin mRNA expression *in vitro* by PCB-77. HepG2 cells (A) and L-02 cells (B) were treated with 0.1 μ M PCB-77 for 24 h, and hepcidin mRNA levels were measured by qRT-PCR ($n = 4$).

Fig. 3. Hepcidin expression and iron content in mice with acute PCB-77 administration. Balb/c mice were i.p. injected with a dose of PCB-77 (80 mg/kg body weight) and were sacrificed at different time points as indicated. Hepatic hepcidin mRNA level (A), serum iron (B), total spleen iron content (C) and total liver iron (D) were then determined ($n = 5$).

class of persistent environmental toxicants, are readily accumulated in liver and fat due to their pronounced feature of lipophilicity (Kodavanti et al., 1998). We here demonstrated that PCB-77 greatly disrupted systemic iron homeostasis by suppressing hepatic hepcidin expression, coupled with increased serum iron and decreased spleen iron. Our group and the other group have found two estrogen response elements (ERE) in the promoter region of hepcidin and described an inhibitory effect of estrogen on hepcidin expression (Hou et al., 2012; Yang et al., 2012). PCBs are endocrine-disrupting chemicals with estrogen-like effects (Kuch et al., 2010), and they could mimic estrogen and disturb the endocrine system and the organs that respond to endocrine signals in organisms (Młynarczuk and Kotwica, 2006). To this end,

we would assume that PCBs inhibit hepatic hepcidin expression through the estrogen-like effects, yet this hypothesis needs further detailed investigation.

Disorders in iron homeostasis are associated with a variety of diseases. The major clinical manifestation of iron deficiency is anemia. In iron deficiency, multiple aspects of cellular functions are compromised, leading to cell growth arrest, differentiation blockade, etc (Ghosh, 2006). On the other hand, iron overload could also cause numerous diseases such as liver cirrhosis (Pietrangelo, 2011). Patients of β -thalassemia (Pennell et al., 2010) and hemochromatosis (Halpern et al., 2009) usually develop severe iron overload due to hepcidin deficiency, and these patients eventually suffer from various disorders

Fig. 4. Changes of hepcidin expression and body iron content after PCB-77 treatment in mice. Mice were sacrificed after PCB-77 administration (80 mg/kg body weight, $n = 5$) for 2 days and 10 days, respectively. (A and E) Relative hepcidin mRNA level. (B and F) Serum iron. (C and G) Total spleen iron. (D and H) Total liver iron.

coupled to excess iron, such as heart failure (Anker et al., 2009). Reduced levels of hepcidin also occur in untreated patients with infection of chronic hepatitis C virus despite ongoing inflammation, leading to predisposition to hepatic iron loading (Drakesmith and Prentice, 2012). Previous studies have shown that high serum levels of PCBs are associated with an increased risk of breast cancer, and PCBs are implicated in promoting breast cancer progression (Aronson et al., 2000). Some epidemiological studies also suggest that PCB accumulation in mammary tissues is linked with high-grade tumors and overall poor prognosis in breast cancer patients (Snedeker, 2001; Woolcott et al., 2001). So far, the mechanisms underlying the correlation between PCB exposure and cancer occurrence and progression are still not understood. Excess iron availability and supply in cells increase the likelihood of gene mutation through production of free radicals and also provide a favorable environment for the growth of tumor cells (Bystrom et al., 2012; Torti and Torti, 2011). A large number of studies have delineated the considerable contribution altered iron metabolism to tumor development (Bystrom et al., 2012; Richardson et al., 2009; Torti and Torti, 2011). For example, case-control surveys of breast cancer patients revealed that intake of meat-derived heme-iron increased the risk of breast cancer (Ferrucci et al., 2009; Kallianpur et al., 2008). Therefore, our current study provides an additional novel mechanism by which PCBs instigate cancer progression through disturbing systemic iron homeostasis.

To summarize, our results demonstrate that PCB-77 significantly suppresses hepatic hepcidin expression *in vitro* and *in vivo* at nontoxic concentrations. PCB-77 greatly disturbs systematic iron homeostasis coupled with increased serum iron and decreased spleen iron concentrations in mice.

Authors' contributions

S.L. and L.W. conceived and designed the study. L.W., S.Z. and L.L. carried out experiments, and analyzed the data. D.Z. and X.L. contributed reagents and materials. L.W. wrote the paper. S.L. revised the article. All authors read and approved the final article.

Conflict of interest

No potential conflicts of interest were disclosed from the authors. There are no non-financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial or any other) to declare in relation to this manuscript.

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