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Effects of polychlorinated biphenyls on liver function and sexual characteristics in Japanese medaka (*Oryzias latipes*)

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ABSTRACT

The effects of polychlorinated biphenyls (PCBs) on liver function and their differences between sexes were analyzed in Japanese medaka (*Oryzias latipes*) exposed to PCB126 or Kanechlor-400 (KC-400) using microarray. PCB exposure induced vitellogenin 1 expression in female medaka while suppressing choriogenin genes, which suggests that the effects of PCBs on estrogen-responsive genes do not occur directly through an estrogen receptor-mediated pathway. Reduction of androgen receptor alpha expression was also observed, and the gene expression pattern in PCB-exposed males changed to become more similar to that of females. Furthermore, changes in glycolysis-related genes indicate that PCB exposure might enhance glucose production via gluconeogenesis in the liver of medaka. Taken together, our results suggest that PCBs disrupt the endocrine system, especially androgen function, and may have the potential to cause demasculinizing effects. Additionally, induction of gluconeogenesis might be a response to maintain glucose levels consumed as a result of PCB exposures.

1. Introduction

Polychlorinated biphenyls (PCBs) are widely distributed in the global environment (Monirith et al., 2003; Ueno et al., 2003). In the past few decades, the concentrations of PCBs in the environment have been decreasing, however, their levels have not recently shown a further declining trend (Ramu et al., 2006, 2007). There are 209 PCB congeners, and their mode-of-action (MoA) depends on the structure of each isomer. For example, it is well known that 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induces a xenobiotic metabolizing enzyme, cytochrome P450 1A, while 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB153) does not (Vezina et al., 2004). As for cytotoxicity or formation of reactive oxygen species, PCB153 has more potential than PCB126 (Lin and Lin, 2006). In addition, their metabolites, hydroxylated PCBs, have also been detected in water, fish, birds and marine mammals (Campbell et al., 2003; Kunisue et al., 2007; Kunisue and Tanabe, 2009; Ueno et al., 2007). Hydroxylated PCBs have largely different chemical characteristics from parental PCBs. Therefore, when evaluating the impacts of PCBs on biota, a wide variety of biological functions should be monitored.

Microarray technology is now becoming a standard tool to monitor the overall responses of organisms to external stimuli, including chemical exposures. Japanese medaka (*Oryzias latipes*) is one of the most suitable organisms to conduct microarray experiments, since a considerable amount of information on genes and toxicological experiments is available for this species. In previous studies, microarrays were used to assess the effects of 17β-estradiol (Kishi et al., 2006) and the acute effects of PCBs (Yum et al., 2010) on medaka. Our group also reported gene expression changes in the brains of medaka exposed to PCBs (Nakayama et al., 2008). Since these studies identified chemical impacts on a wide variety of biological functions, global gene expression analysis using microarrays is one of the best approaches to elucidate the effects of chemicals with various MoAs.

In the present study, we analyzed the alteration of gene expression profiles in the livers of both male and female Japanese medaka exposed to PCBs, since there is currently a need for more information about their transcriptomic responses to PCBs in order to better understand MoAs of PCBs. To analyze the responses to PCB exposures, we selected Kanechlor 400 (KC-400), a major industrial product of PCBs, and PCB126 that was a coplanar PCB and a major component of KC-400. We also compared the gene expression profiles between males and females to identify sex-specific expression patterns that can be used to evaluate the effects of PCB exposure on sexual characteristics.

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2. Materials and methods

2.1. Preparation of test diets

A mixture of PCBs (KC-400) and PCB126 were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and AccuStandard (New Haven, CT), respectively. To prepare test diets containing KC-400 or PCB126, 5 g of freeze-dried brine shrimp flakes (TetraDelica; Tetra Japan, Tokyo, Japan) were wetted with 2 ml of an ethanol solution containing 165 μg of KC-400 or 1.65 μg of PCB126, then dried with a blow-drier to yield test diets containing 33 μg/g of KC-400 or 0.33 μg/g of PCB126. For the control diet, 5 g of TetraDelica was wetted with 2 ml of ethanol and dried with a blow-dryer.

2.2. Exposure conditions

The female leukophore free-II (FLF-II) strain of medaka was provided by Prof. Yuko Wakamatsu (Nagoya University, Nagoya, Japan). Sexually mature medaka (six months post-hatch) was randomly selected from a brood stock maintained for a few years in our laboratory. Three fish of each sex were placed in each 2.5-L glass chamber, and two replicate chambers (six fish: three males and three females) were prepared for each treatment group. Culture water was prepared from diluted artificial seawater (salinity was adjusted to 0.1%) as in our previous report (Nakayama et al., 2005). Water temperature was kept at 23 ± 1 °C for the whole exposure period by adjusting the room temperature. The fish were kept under a constant 16:8-h light:dark photoperiod and fed with *Artemia* nauplii (≤24 h after hatching) twice a day. They were acclimated for one week under these conditions.

Half the water in each chamber was replaced with fresh culture water each day. Each of the test diets was fed (at 3% of body wt [b.w.]) to each group of fish so that they received 1 μg KC-400/g b.w. or 0.01 μg PCB126/g b.w. daily. Fish in the control group received the control diet at 3% of body weight daily. Fish receiving the test diets were fed in the morning and afternoon. *Artemia* nauplii also were fed to fish of each group in the evening. Neither adverse effects on morphology nor mortality were observed in the fish during the three-week exposure period.

2.3. Microarray experiment

After the exposure period, three males and three females in each treatment group were dissected and the livers were collected. The liver samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. RNeasy Mini Kit (QIAGEN, Valencia, CA) was used for the extraction and purification of medaka liver total RNA, and 2 μg each of the extracted total RNA was reverse-transcribed with T7-oligo dT primer. Subsequently, double-stranded cDNA was synthesized and transcribed *in vitro* with amino-allyl UTP to generate amino-allyl labeled aRNA probe samples. The purified aRNA probe samples were then coupled with amine reactive Cy5, and it was re-purified for hybridization on a cDNA microarray. All of the processes from reverse-transcribing the total RNA to aRNA labeling and purification were carried out with Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX).

A cDNA microarray containing 741 medaka genes was customized and fabricated by Ecogenomics (Kurume, Japan) for use in this study. These target genes were PCR-cloned using gene-specific primer sets from the liver and brain of adult medaka. After purification, each gene was spotted three times (2223 probes in total) on APS (amino propyl silane)-coated microarray slides (Matsunami Glass, Osaka, Japan) in 50% DMSO. The target-spotted microarrays went through 150 mJ/cm^2-UV-cross-linking and 80 °C-baking processes for immobilization, followed by treatment with succinic anhydride (6.6 g)/1-methyl-2-pyrrolidone (400 ml) blocking reagent at 42 °C for 20 min and pre-hybridization washes (one wash in 1× SSC/0.2% SDS for 2 min, two washes in 0.1× SSC/0.2% SDS for 2 min each, then one rinse in ultra-pure water for 10 s). Hybridization of the labeled cRNA probe samples and the target genes on the microarray was carried out for 16 hours at 42 °C in 45 μl of 50% formamide (Wako, Osaka, Japan)/5× SSC (SIGMA, St. Louis, MO)/0.5% SDS (Ambion) hybridization solution in a moisture chamber, followed by post-hybridization washes (two washes in 1× SSC/0.2% SDS at 42 °C for 5 and 15 min, two washes in 0.1× SSC/0.2% SDS at ambient temperature for 5 min each, then final two washes in 0.1× SSC at ambient temperature for 2 min each). The process-completed microarray slides were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) at 10 μm resolution.

2.4. Data analyses

The digitalized fluorescent intensities were logarithmically transformed and normalized by the expression levels of a housekeeping gene, acidic ribosomal phosphoprotein PO. The differences in expression levels of each gene among treatment groups in each sex were analyzed by one-way analysis of variance (ANOVA). The sexual differences in gene expression of control males and females were also analyzed by Student's *t*-test. We then applied a principal component analysis (PCA) to the gene expression values that showed significant differences by one-way ANOVA or *t*-test, and obtained principal component (PC) scores of each individual and correlation coefficients between each probe and each PC. All statistical analyses were conducted using the R project language (http://www.r-project.org/).

3. Results and discussion

Microarray data analyses revealed that the expression data of 826 or 837 out of 2223 probes in males or females, respectively,

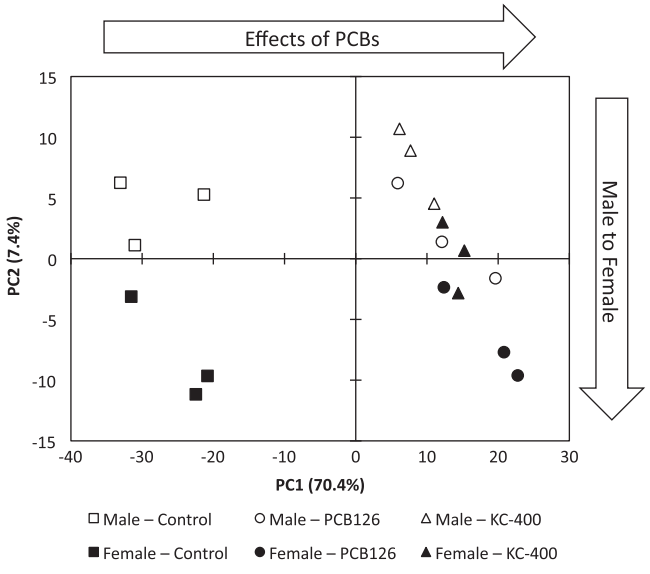

Fig. 1. Principal component scores calculated from the gene expression of the selected 546 probes whose expressions were significantly different (≥1.5-fold compared to control) among the three treatment groups in either male or female medaka ($n = 3$). The probes were screened by one-way analysis of variance ($p < 0.05$), and the statistical analyses were conducted independently in males and females. The horizontal and vertical axes represent the first and second principal components (PC1 and PC2), respectively. PC1 and PC2 appear to reflect the effects of PCB exposures and sexual characteristics, respectively.