Changes in Differentially Expressed Genes in the Liver of *Oryzias latipes* by Binary Exposure to Carcinogenic Polycyclic Aromatic Hydrocarbons

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Abstract − The biological effects of carcinogenic polycyclic aromatic hydrocarbons (cPAHs) including benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBA), benzo[a]anthracene (BaA), benzo[b] fluoranthene (BbF), benzo[k]fluoranthene (BkF), and indeno[1,2,3-c, d]pyrene (InP) on transcriptomic changes were determined in the liver of *Oryzias latipes*. Differentially expressed genes (DEGs) by binary exposure to cPAHs (BaP+BaA, BaP+BbF, BaP+BkF, BaP+DbA, BaP+InP) were screened by annealing control primers-based polymerase chain reaction followed by sequence analysis and BLAST searching. The results showed that four DEGs were commonly expressed by cPAHs and they were identified as ribosomal protein S4, coagulation factor II, elongation factor 1 beta, and a predicted protein similar to human immunodeficiency virus type I enhancer binding protein 3. This finding suggests that binary exposure to cPAHs interferes protein synthesis required for fundamental liver functions in fish.

Key words : organic pollutant, transcription, medaka, aquatic pollution

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitously found in air and aquatic environments as a result of the incomplete combustion of organic materials from industrial processes and human activities (Hylland 2006). PAHs prefer to associate with particulate matter in seawater or marine sediments due to their liphophilic and hydrophobic characteristics, which is known to be accumulated in marine organisms throughout food web (Adamo *et al.* 1997; Baumard *et al.* 1998). Among them, benzo[a]pyrene (BaP), dibenzo [a,h]anthracene (DBA), benzo[a]anthracene (BaA), benzo [b]fluoranthene (BbF), benzo(k)fluoranthene (BkF), and indeno[1,2,3-c, d]pyrene (InP) were known as carcinogenic PAHs (cPAHs) due to their potent carcinogenic and mutagenic activities (IARC 1984).

It is widely accepted that aquatic contamination is caused by the mixture of various organic compounds. These toxic compounds cause the onset of biological disorders associated with development and reproduction of aquatic organisms by interacting with target macromolecules such as proteins or nucleic acids (Guillette *et al.* 1995; Fielden and Zacharewski 2001; Waring *et al.* 2001; Aravindakshan *et al.* 2004; Moggs 2005; Williams *et al.* 2007). Although cytochrome P450 (CYP450) family has been applied to evaluate the toxicological effects of PAHs on fish in field and laboratory studies, risk assessments for the compounds remain elusive (Peters *et al.* 1997; Gravato and Santos 2002; Nacci *et al.* 2002; Carlson *et al.* 2004; Greytak *et al.* 2005; Hoffmann *et al.* 2006; Jonsson *et al.* 2006; Patel *et al.* 2006). Thus, iden-

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Table 1. Test conditions of binary exposure to cPAHs in the liver of *O. latipes*

Parameters	Conditions		
	Control	Each cPAH	BaP+other cPAHs
Exposure type	Water-borne	〃	〃
Test organism	*Oryzias latipes*	〃	〃
Weight (g)	0.14±0.04	0.31±0.09	0.39±0.07
Length (cm)	2.84±0.22	3.22±0.48	3.30±0.17
Exposure duration	24 hrs	〃	〃
pH	7.84±0.02	7.82±0.01	7.81±0.02
Dissolved oxygen (mg L^{-1})	5.39±0.09	5.55±0.13	5.50±0.07
Salinity (psu)	34.51±0.08	34.70±0.08	34.75±0.17
Temperature (°C)	24.68±0.01	24.81±0.13	24.91±0.08
Photoperiod	16 Light : 8 Dark	〃	
Feeding regime	None	〃	
Test water	Filterated seawater (0.22 μm cartridge filter)	〃	

Value shown are mean±S.D., n=7.
cPAHs: BaP, benzo[a]pyrene; DBA, dibenzo[a,h]anthracene; BaA, benzo[a]anthracene; BbF, benzo[b]fluoranthene; BkF, benzo(k)fluoranthene; InP, indeno [1,2,3-c, d]pyrene

tification of altered genes in fish using genomic analysis would be a way to assess the biological effects of the compounds on marine environment.

In the present study, the differentially expressed genes (DEGs) were identified to investigate the effects of cPAHs on the change of transcripts in the liver of *Oryzias latipes* (*O. latipes*). The DEGs were screened by annealing control primer (ACP)-based polymerase chain reaction (PCR) with 20 arbitrary primers because the ACP system has advantage to improve the specificity of PCR amplification and the elimination of false-positive results (Hwang *et al*. 2003; Kim *et al*. 2004). *O. latipes* (Japanese medaka) was used as a model species in this study because it has several advantages for laboratory study. For instance, it is small in size, easy to rear in a laboratory, and has a short life cycle. The fish also can adapt to a wide range of saline condition, although it is a freshwater species (Sakamoto *et al*. 2001; Inoue and Takei 2003).

MATERIALS AND METHODS

1. Animal maintenance and chemical exposure

O. latipes was maintained at 25°C under a constant photoperiod of 16 : 8 hrs (light : dark). Water quality was monitored by measuring pH, dissolved oxygen, and temperature (Table 1). During acclimation in seawater, the fish was fed newly hatched brine shrimp and a commercial flake food twice a day, but the experiments were performed without being fed. All chemicals were purchased from Sigma-Aldrich (MO, USA). DMSO was used as a solvent control. Male medaka adapted to seawater was exposed to each of cPAHs (BaP, DBA, BaA, BbF, BkF, InP) or binary combination of cPAHs for 24 hrs. The ratio of binary combination was 1 : 1 based on BaP concentration (25 μg L^{-1}). Following water-borne exposure, the fish was sacrificed and the head was immediately frozen in liquid nitrogen and then stored at −80°C until RNA isolation.

2. Total RNA isolation and first-strand cDNA synthesis

Total RNAs were isolated from the liver of *O. latipes* using easy-spin™ [DNA free] Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi, Korea). Briefly, pooled liver samples (n=7) was homogenized in 1 mL of lysis buffer and then vigorously vortexed in room temperature for 10 sec. After adding 200 μL of chloroform, the solution was centrifuged at 13,000 rpm for 10 min at 4°C. Then the supernatant (400 μL) was transferred to a fresh 1.5 mL tube and 400 μL of binding buffer was added and mixed it well by 2~3 times gentle inverting. The supernatant was loaded to the column and centrifuged at 13,000 rpm for 30 sec. Following discarding the flow-through, the column was washed by adding washing buffer and centrifuged at 13,000 rpm for 1~2 min to dry the column membrane. The 50 μL of Elution buffer was added directly onto the membrane after the

column was placed in a new 1.5 mL tube. Finally, the column was incubated for 1 min at room temperature and centrifuged 13,000 rpm for 1 min to elute total RNAs. The isolated total RNAs were used for the synthesis of the first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 hrs at 42°C in a final reaction volume of 20 μL containing 3 μL of the purified total RNAs, 4 μL of 5 X reaction buffer (Promega, WI, USA), 5 μL of dNTPs (each 2 mM), 2 μL of 10 μM dT-ACP1 [5′-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3′], 0.5 μL of RNasin® RNase Inhibitor (40 U μL^{-1}; Promega), and 1 μL of moloney murine leukemia virus reverse transcriptase (200 U μL^{-1}; Promega). The first-strand cDNAs were diluted by the addition of 80 μL of ultra-purified water for the ACP-based PCR, and stored at −20°C until used.

3. ACP-based PCR

DEGs were screened by ACP-based PCR method (Kim *et al.* 2004) using the GeneFishing™DEG kits (Seegene, Seoul, South Korea). Briefly, the second-strand cDNA synthesis was conducted at 50°C during one cycle of the first-stage PCR in a final reaction volume of 20 μL containing 3∼5 μL (about 50 ng) of diluted first-strand cDNA, 1 μL of dT-ACP2 (10 μM), 1 μL of 10 μM arbitrary ACP, and 10 μL of 2× Master Mix (Seegene). The PCR protocol for the second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 sec, followed by 65°C for 40 sec, 72°C for 40 sec, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidiumbromide. The differentially expressed band was selected as DEGs based on the size of PCR products (300∼1,200 base pairs), intensity (at least more than two folds compared with control), and shape (sharp but not smear).

4. Direct sequencing

The differentially expressed bands were re-amplified and extracted from the gel by using the GENCLEAN®II Kit (Q-BIO gene, CA, USA), and directly sequenced with ABI PRISM®3100-*Avant*Genetic Analyzer (Applied Biosystems, CA, USA) using universal primer (5′-GTCTACCAGGCATTCGCTTCAT-3′).

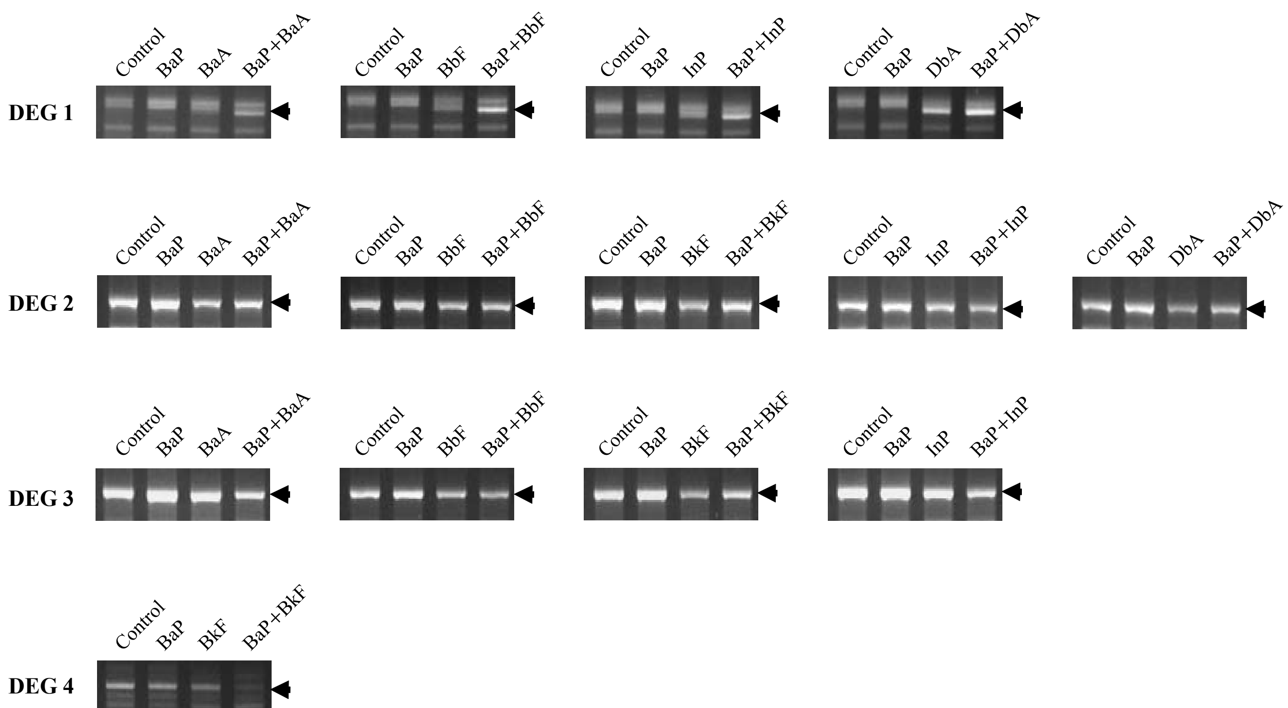

Fig. 1. Identified DEGs in the liver of Japanese medaka were selected by comparing density of bands produced by the control groups (see arrows). DEG 1 was upregulated by all binary exposure to cPAHs excepted BaP+BkF, whereas DEG 2, 3, and 4 were downregulated. Interestingly, DEG 4 was downregulated by binary exposure to BaP+BkF only.

Table 2. Identified DEGs by binary exposure to cPAHs in the liver of *O. latipes*

DEGs	Up- or down-regulation	Identities
1. ribosomal protein S4 [*Solea senegalensis*]	Up	41/44 (93%)
2. coagulation factor II [*Danio rerio*]	Down	37/41 (90%)
3. elongation factor 1 beta [*Oryzias latipes*]	Down	39/46 (84%)
4. PREDICTED similar to human immunodeficiency virus type I enhancer binding protein 3 [*Danio rerio*]	Down	24/58 (41%)

Table 3. Commonly identified DEGs by binary exposure to cPAHs in the liver of *O. latipes*

Groups	DEG 1	DEG 2	DEG 3	DEG 4
BaP+BaA	●	●	●	
BaP+BbF	●	●	●	
BaP+BkF		●	●	●
BaP+InP	●	●	●	
BaP+DbA	●	●		

5. Statistical analysis

The difference of an expression level between each group was determined by their density on agarose gel compared with control (mean $\pm$ SD). The statistical significance was analyzed with the student's *t*-test using GraphPad Prism 4 (GraphPad Software Incorporation, CA, USA) and set at $p < 0.05$.

RESULTS AND DISCUSSION

This study was conducted to understand the changes in DEGs by acute binary exposure to cPAHs in the liver of Japanese medaka. The results demonstrated that four DEGs were regulated by following binary exposure to cPAHs, such as BaP+BaA, BaP+BbF, BaP+BkF, BaP+DbA, and BaP+InP, and they were identified as ribosomal protein S4, coagulation factor II, elongation factor 1 beta, and a predicted protein similar to human immunodeficiency virus type I enhancer binding protein 3 (Fig. 1) (Tables 2, 3).

DEG 1 was found to be a protein that is highly homologous with ribosomal protein S4 in *Solea senegalensis* and was upregulated by all binary exposures to cPAHs excepted BaP+BkF. It is well known that ribosomal protein plays a critical role in protein biosynthesis by interacting with translational apparatus including ribosomal RNA (Lodish *et al.* 2003). Although genetic information stores protein information, translation is a critical step for the production of functional proteins that contribute to the variety of biological activities (Kapp and Lorsch 2004). For this reason, transcriptomic changes of ribosomal proteins may alter the efficient assembly of each ribosome and influence protein biosynthesis (Liebman *et al.* 1995). Interestingly, DEG 3 that was downregulated by cPAHs was found to be a protein having structural similarity to the elongation factor 1 beta (EF-1β) in *O. latipes* involved in the elongation step of protein synthesis (Riis *et al.* 1990). In eukaryotic cell, EF-1 is composed of 4 subunits (α, β, γ and δ) and the interaction of each subunit helps continuous elongation steps. In particular, the EF-1β, a guanine nucleotide exchange factor, is essential for cell growth and plays an important role in translational rate and fidelity (Hiraga *et al.* 1993; Carr-schmid *et al.* 1999).

DEG 2 was found to be a downregulated protein that is highly similar to coagulation factor II (prothrombin) in *Danio rerio* involved in hemostasis. Previous studies have shown that the deficiency of prothrombin in the development of mouse causes embryonic lethality (Sun *et al.* 1998). In addition, cPAHs also interfere with blood coagulation by suppressing prothrombin gene expression in the liver, and causes inflammation by interrupting the aggregation of platelet (Coughlin *et al.* 1992).

Taken together, these findings suggest that cPAHs in the liver of Japanese medaka hinder protein synthesis by preventing accurate elongation step as well as blood coagulation, and thus causes liver dysfunctions in fish. Although limited primers were used in this study, identified genes may provide an informative insight into assess marine environmental risk of cPAHs.

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