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Alterations in Differentially Expressed Genes in the Head of *Oryzias latipes* Following Benzo[a]pyrene Exposure

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Abstract Differentially expressed genes (DEGs) in the head of medaka (*Oryzias latipes*) were investigated to assess the biological effects of benzo[a]pyrene (BaP) on genomic alterations. Eight and ten DEGs were identified in response to short (48 h) and long-term (15 days) exposure to BaP (25 μg/L), respectively, using annealing control primer-based polymerase chain reaction. Among the DEGs, matrilin 1 and prothrombin were commonly detected, suggesting that BaP exposure can alter the expression of genes associated with the formation of extracellular matrix and blood coagulation in the head of medaka.

Keywords Polycyclic aromatic hydrocarbon · Environmental pollutant

Polycyclic aromatic hydrocarbons (PAHs), which are formed as a result of the incomplete combustion of organic materials during industrial processes and human activities, are ubiquitous in air and aquatic environments (Hylland 2006). PAHs tend to associate with particulate materials in seawater or marine sediments due to their liphophilic and hydrophobic characteristics. As a result, these materials have accumulated in marine organisms through the food web (Adamo et al. 1997). Benzo[a]pyrene (BaP) is known as the most toxic compound among PAHs due to its potent carcinogenic and mutagenic activities (IARC 1987). Aquatic organisms are often exposed to multiple anthropogenic pollutants including PAHs, which can lead to disorders associated with their development and reproduction via genomic changes (Aravindakshan et al. 2004; Guillette et al. 1995).

In this study, *Oryzias latipes* (*O. latipes*) was used as a model species for a laboratory study because the fish is small in size, easy to rear and has a short life cycle. *O. latipes* can be a test animal for marine ecotoxicological effects because the fish is able to adapt to a wide range of saline conditions even though it is a freshwater species (Inoue and Takei 2003; Park et al. 2005). Identification of genes altered in fish using genomic analysis would be an effective method of assessing the biological effects of toxic compounds on marine organisms. Annealing control primer (ACP) is a new differential display-polymerase chain reaction (PCR) technique developed to identify differentially expressed genes (DEGs) under various physiological conditions. The technology improves the specificity of PCR amplification by eliminating false-positive results due to the specific primers controlling the annealing temperatures (Kim et al. 2004). Therefore, in this study, DEGs were identified in the head of *O. latipes* that had been exposed to BaP by ACP-based PCR using 120 arbitrary primers.

Materials and Methods

O. latipes was gradually adapted to a high saline condition according to the previous study (Inoue and Takei 2003). Briefly, brackish water was made first by mixing a half of freshwater (20 L) and same volume of seawater. Then a

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half of the water was replaced with same volume of seawater every day for 15 days. The fish was fed newly hatched brine shrimp (<24 h after hatching) and commercial flake food twice a day. There was no mortality observed during acclimation in seawater over 1 month. The fish were maintained at 25°C under a constant photoperiod of 16: 8 h (light: dark) and the water quality was monitored by measuring the pH, dissolved oxygen, and temperature (Table 1). The F3 generation of *O. latipes* adapted to seawater was used for exposure experiments.

Unlike the long-term experiment, the short-term experiment was performed on fish that were not fed. All chemicals used for the experiment were purchased from Sigma–Aldrich (St. Louis, MO, USA). A minimal concentration of dimethyl sulfoxide (<0.001%) was used as a vehicle to prevent cellular damage to the fish. Male medaka that had adapted to seawater were exposed to BaP (25 μg/L) which was replaced every day with fresh-made one for 48 h or 15 days. Following waterborne exposure in seawater, the fish were sacrificed and the heads including a gill were removed and immediately frozen in liquid nitrogen. The samples were then stored at −80°C until RNA isolation.

The total RNA was isolated from the heads of *O. latipes* using an easy-spin ™[DNA free] Total RNA Extraction Kit (iNtRON Biotechnology, Kyunggi, Korea). The isolated total RNA was used to synthesize the first-strand cDNA using dT-ACP1 by reverse transcriptase. Screening of the DEGs was then conducted using an ACP-based PCR method (Kim et al. 2004) with the GeneFishing™DEG kits (Seegene, Seoul, Korea). The 120 sets of arbitrary primers were used and each ACP-based PCR was performed in duplicate. Briefly, the second-strand cDNA synthesis was conducted at 50°C during one cycle of the first-stage PCR in a mixture with 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 s followed by 65°C for 40 s, 72°C for 40 s and a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel containing ethidium bromide. The differentially expressed bands were selected as DEGs based on their size (300–1,200 base pairs), intensity (a change of at least two folds when compared with the control) and the shape (sharp but not smeared) of the PCR products.

The differentially expressed bands were re-amplified and extracted from the gel using the GENCLEAN®II Kit (Q-BIO gene, CA, USA), after which they were directly sequenced using an ABI PRISM®3100-*Avant*Genetic Analyzer (Applied Biosystems, CA, USA) and a universal primer (5′-GTCTACCAGGCATTCGCTTCAT-3′). Quantitative real-time PCR was performed in triplicate in 384-well plates (ABgene, Hamburg, Germany) using *Power* SYBR® Green PCR Master Mix with the ABI Prism 7,900 Sequence Detection System (Applied Biosystems). The primers used for real-time PCR are given in Table 2.

All data were expressed as the means ± S.D. Data were statistically analyzed by a two-tailed homoscedastic Student's *t*-test unless otherwise indicated. A *p*-value of less than 0.05 was regarded as statistically significant.

Results and Discussion

Following short and long-term exposure to BaP, 8 and 10 DEGs were identified (Table 3, Fig. 1), respectively. Specifically, following long-term exposure, 4 DEGs (# 6, 10, 14, 16) were found to have no significant similarity and several groups of DEGs [(# 2, 12, 17) (# 4, 5), and (# 1, 13)] were identified as the same genes.

Interestingly, matrilin 1 and coagulation factor II (prothrombin) were detected in response to both short and long-term exposure to BaP. The expression of these DEGs was confirmed by real-time PCR and normalized to 18S rRNA levels as an internal control. The primers used in this study were presented in Table 2.

The results showed that matrilin 1 mRNA was upregulated by short-term exposure to BaP ($p < 0.001$), whereas

Table 1 Test conditions for short and long-term exposure to BaP in the head of *O. latipes*

	Short-term (48 h)		Long-term (15 days)	
	Control	BaP	Control	BaP
Weight (g)	0.14 ± 0.04	0.18 ± 0.04	0.27 ± 0.08	0.30 ± 0.12
Length (cm)	2.84 ± 0.22	2.34 ± 0.13	3.05 ± 0.26	3.07 ± 0.34
pH	7.74 ± 0.12	7.77 ± 0.15	7.88 ± 0.07	7.90 ± 0.05
D.O. (mg/L)	5.02 ± 0.77	4.94 ± 0.79	5.95 ± 0.17	5.80 ± 0.24
Salinity (psu)	34.69 ± 0.30	34.67 ± 0.22	34.92 ± 0.57	34.63 ± 0.22
Temperature (°C)	25.09 ± 0.10	25.03 ± 0.03	24.11 ± 0.29	24.47 ± 0.20

Table 2 Primers used for real-time PCR in the head of *O. latipes*

Genes		Primers
Matrilin 1	Forward	5′-GCCTTGCTGGAGAACAAGAT-3′
	Reverse	5′-TTTGCACAAACACACACACG-3′
Prothrombin	Forward	5′-GCTGGTATCAGATCGGCATT-3′
	Reverse	5′-AATGCTGGATTTCTGTTGGG-3′
18S rRNA	Forward	5′-CCTGCGGCTTAATTTGACTC-3′
	Reverse	5′-AACTAAGAACGGCCATGCAC-3′

long-term BaP exposure downregulated the expression of matrilin 1 mRNA (Fig. 2a). The downregulation of matrilin mRNA in response to long-term BaP exposure may be a result of the desensitization of receptors binding for BaP against repeated stimulation of BaP. Desensitization would be a possible mechanism for the protection of continuous genomic changes in response to chronic stimulation, which minimizes the effect of the compound to which the organism is being exposed, although we currently do not have experimental evidence with regard to the present data. Matrilin 1 is a major component of extracellular matrix that is involved in the formation of nonarticular cartilage (Wagener et al. 2005) that covalently binds to aggrecan and also forms collagen-dependent and independent filamentous networks in the extracellular matrix (Wagener et al. 2005). In addition, mammalian matrilin 1 plays an important role in the proliferative stage during chondrogenesis (Muratoglu et al. 1995; Wagener et al. 2005). Taken together, these data suggest that the expression of matrilin 1 mRNA regulating extracellular matrix in chondrogenesis is finely controlled by the duration of BaP exposure, although dual mechanisms underlying matrilin 1 gene expression and functions in fish remain elusive.

Unlike matrilin 1, expression of prothrombin mRNA was upregulated by both short and long-term exposure to BaP ($p < 0.001$), and the level of the mRNA was much higher in response to long-term exposure than in response to short-term exposure (Fig. 2b). The fact that both short and long-term exposure to BaP upregulated prothrombin mRNA expression indicates that 15 days of repeated stimulation with BaP may not be necessary to induce desensitization of prothrombin binding when compared with the expression of matrilin 1. It is well known that

Table 3 DEGs identified following short and long-term exposure to BaP in the head of *O. latipes*

	DEG #	Genes	Identities
Acute	1	Fructose-bisphosphate aldolase A [*Oryzias latipes*]	42/43 (97%)
	2	Prostaglandin D2 synthase [*Danio rerio*]	30/41 (73%)
	3	Alpha-1-antitrypsin [*Pseudopleuronectes americanus*]	19/25 (76%)
	4	Matrilin 1 [*Danio rerio*]	27/32 (84%)
	5	Prothrombin [*Oncorhynchus mykiss*	82/176 (46%)
	6	Thiopurine methyltransferase [*Danio rerio*]	32/44 (72%)
	7	Fibrinogen beta chain precursor [*Paralichthys olivaceus*]	40/40 (100%)
	8	Lens intrinsic membrane protein 2.1 [*Danio rerio*]	33/37 (89%)
Chronic	1	Coagulation factor II (prothrombin) [*Danio rerio*]	41/45 (91%)
	2	Apolipoprotein 14 kDa [*Oplegnathus fasciatus*]	28/41 (68%)
	3	Betaine-homocysteine methyltransferase [*Danio rerio*]	36/42 (85%)
	4	Warm-temperature-acclimation-related-65 [*Oryzias latipes*]	43/52 (82%)
	5	Warm-temperature-acclimation-related-65 [*Oryzias latipes*]	93/172 (54%)
	6	No significant similarity found	
	7	Alpha-1-antitrypsin [*Pseudopleuronectes americanus*]	31/38 (81%)
	8	C1 inhibitor [*Oncorhynchus mykiss*]	25/33 (75%)
	9	Transferrin [*Oryzias latipes*]	46/69 (66%)
	10	No significant similarity found	
	11	Matrilin 1 [*Danio rerio*]	27/32 (84%)
	12	Apolipoprotein A1 [*Fundulus heteroclitus*]	28/36 (77%)
	13	Coagulation factor II [*Oncorhynchus mykiss*]	81/175 (46%)
	14	No significant similarity found	
	15	Eukaryotic translation elongation factor 2 [*Danio rerio*]	54/82 (65%)
	16	No significant similarity found	
	17	Apolipoprotein AI precursor [*Platichthys flesus*]	28/41 (68%)
	18	Uridine phosphorylase 2 [*Danio rerio*]	29/38 (76%)

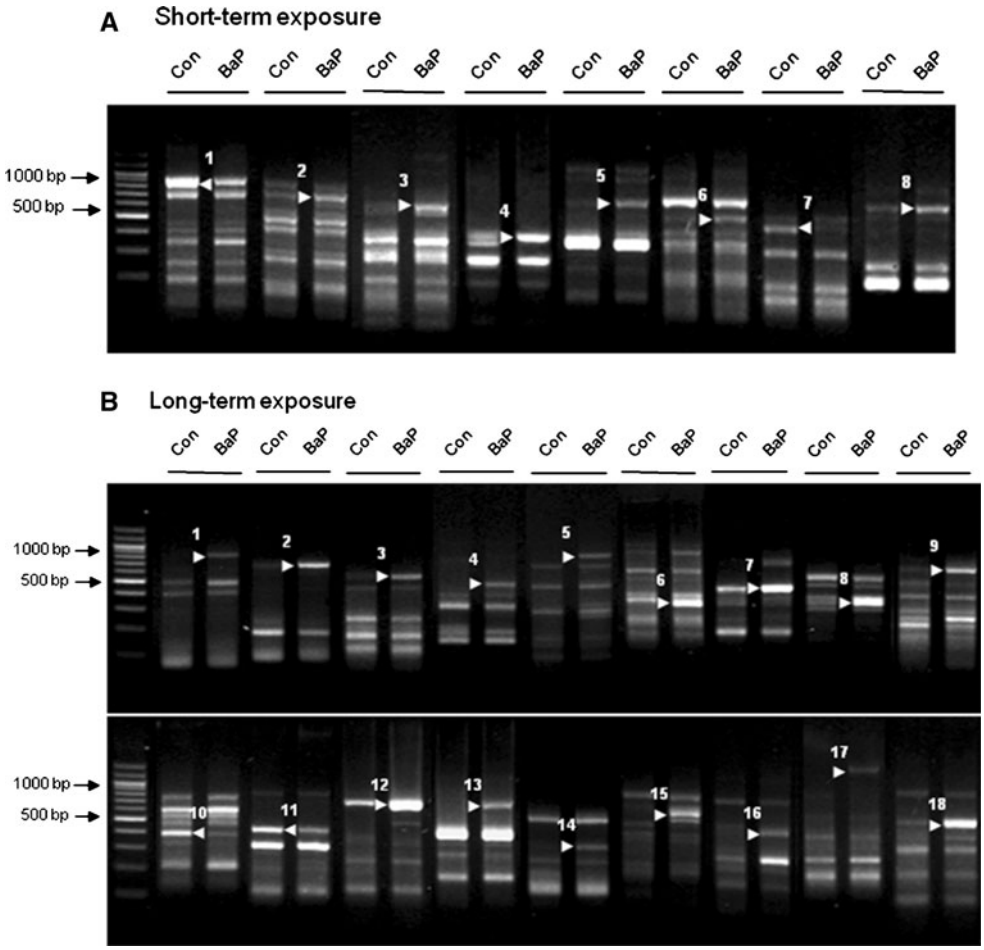

Fig. 1 Identification of DEGs in the head of *O. latipes* following short (48 h) (**a**) and long-term (15 days) (**b**) exposure to BaP. Differentially expressed bands were detected by comparing the density of bands produced by the control groups. Con, control

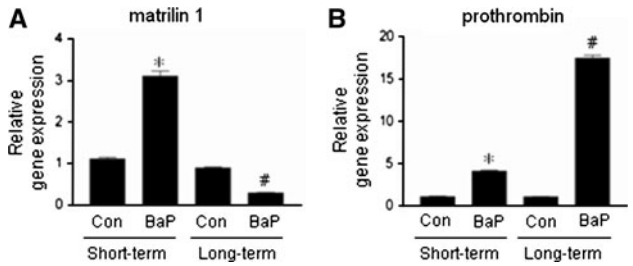

Fig. 2 The expression of matrilin 1 (**a**) and prothrombin (**b**) mRNAs in the head of *O. latipes* following BaP exposure. The expression levels of target genes were quantified by real-time PCR and normalized to 18S rRNA as an internal control (Con). The expression of matrilin 1 was upregulated in response to short-term exposure to BaP, whereas it was downregulated following long-term exposure. The expression of prothrombin was upregulated by both short and long-term exposure to BaP, with a higher degree of upregulation being observed in response to long-term exposure. *, #$p < 0.001$ vs. control groups

prothrombin, a precursor of thrombin that plays a key role in the conversion of fibrinogen to fibrin and platelet activation, is critical for the process in the coagulation cascade of blood (Degen and Sun 1998; Walsh 2007). A previous study showed that thrombin production is stimulated by the formation of fibrin clotting (Butenas and Mann 2002). In this study, BaP exposure was found to increase the expression of prothrombin mRNA in the head of medaka. With limited information, it is possible to predict that exposure to BaP can increase the induction of fibrin clotting, which promotes blood coagulation by enhancing prothrombin mRNA expression as seen in this study. The data generated by this study may help provide insight into the risks associated with the exposure of marine organisms to BaP, although identification of the molecular mechanisms underlying the changes induced in fish by BaP exposure must still be identified.

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