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Characterization of Protein Expression in the Head of *Oryzias latipes* in Response to Acute and Chronic Exposure to Benzo(a)pyrene

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Abstract – In this study, alterations in whole proteome expression patterns in the head of Japanese medaka (*Oryzias latipes*) was investigated following acute or chronic exposure to benzo(a) pyrene (BaP) (25 μg L^{-1}) for 48 hrs and 15 days, respectively. The results showed that 9 and 6 protein spots were statistically different, relative to controls, in response to acute and chronic BaP exposure, respectively. In the acute exposure group, 5 spots were upregulated and 4 spots were downregulated, while in the chronic exposure group, 4 spots were upregulated and 2 spots were downregulated. Three of these spots were common to both the acute and chronic BaP exposure groups and were identified using LC-MS/MS followed by database searching. These 3 spots were found to be associated with structural proteins belonging to the actin and keratin families. These data suggest that acute and chronic exposures to BaP may affect tissue morphology in the head of Japanese medaka.

Key words : Japanese medaka, benzo(a)pyrene, proteome, environmental pollution

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are produced by incomplete combustion of organic materials and are released into marine environments through various routes. They prefer to adhere to particles in seawater or sediments due to their liphophilic and hydrophobic characteristics, which in turn could affect benthic organisms living in close relationship with the ground. Throughout the food web, organic chemicals can be bioaccumulated and biomagnificated in lipid rich tissues of marine organisms, which could adversely affect the physiology of many marine organisms including fish. Benzo(a)pyrene (BaP) is known as a highly toxic chemical among the PHAs and has a potent carcinogenic and mutagenic activity. For instance, BaP-7,8-dihydrodiol-9,10-epoxide, the intermediate of BaP generated by cytochrome P4501A, is thought to be the ultimate carcinogenic metabolite of BaP and plays an important role in BaP-induced carcinogenesis by aryl hydrocarbon receptor signaling cascades (Bartsch 1996; Shimizu *et al.* 2000; Walker 2001). Previous studies often use the cytochrome P450 (CYP450) family as biomarkers to evaluate the toxicological effects of PHAs on fish during field and laboratory studies because the CYP450 monooxygenase activity is affected by PAHs (Peters *et al.* 1997; Carlson *et al.* 2002, 2004; Gravato and Santos 2002, 2003; Nacci *et al.* 2002; Greytak *et al.* 2005; Hoffmann *et al.* 2006; Jonsson *et al.* 2006; Patel *et al.* 2006). The toxicity of pollutants is believed to be caused by protein dysfunctions that results from the mixture of different pollutants in the environment, although supporting evidence for this hypothesis is not yet available.

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Thus, the characterization of altered proteins in aquatic organisms using proteomic analysis is an alternative approach to understanding the toxicological risk of organic pollutants. Although protein information regarding aquatic organisms remains to be fully developed, proteomic analysis is a powerful tool for evaluating the biological effects of organic pollutants in fish.

In this study, Japanese medaka (*O. latipes*) was used as a model species because of its numerous advantages. For instance, it is small in size, easy to propagate in the laboratory, and has a short life cycle. The fish also can adapt to a wide range of saline conditions, although it is a freshwater species (Sakamoto *et al.* 2001; Inoue and Takei 2003). For these reason, Japanese medaka has been used as a test organism for marine ecotoxicological risk assesement (Park *et al.* 2005). The present study was designed to investigate altered protein expression in the head of Japanese medaka adapted to seawater using proteomic analysis so as to understand the biological effects of BaP on marine organisms.

MATERIALS AND METHODS

1. Animal maintenance and chemical exposure

Japanese medaka were purchased from Greenfish (Seoul, Korea) and 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). The fish were fed newly hatched brine shrimp and a commercial flake food twice a day. Male medaka that were adapted to seawater were exposed to 25 μg L^{-1} BaP (Sigma-Aldrich, MO, USA) or DMSO (Sigma-Aldrich) as a solvent control for 48 hrs and 15 days, respectively. During acute exposure, the fish was exposed to BaP without being fed. Following waterborne exposure, the fish were sacrificed and the head including the gill was removed and then immediately frozen in liquid nitrogen and stored at −80°C until protein extraction.

2. Protein extraction

Head samples were homogenized in lysis buffer [7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% (w v^{-1}) (3-cho) amidoprophy-dimethy-ammon-1 proponesulfonate (CHAPS) (USB, OH, USA), 2% (v v^{-1}) Pharmalyte pH 3～10 (Amersham Biosciences, Uppsala, Sweden), and 40 mM dithiothreitol (DTT) (Amersham Biosciences)] and incubated for 1 hr at 4°C. The lysate was then centrifuged at 13,000 rpm for 30 min at 4°C and the supernatant was transferred to a new 1.5 mL microcentrifuge tube. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA) and samples were stored in aliquots at −80°C until further analysis.

3. Two-dimensional electrophoresis (2-DE) and image analysis

Seven-centimeter Immobiline DryStrip gels, pH 3～10 (Amersham Biosciences), were rehydrated in the Immo-

Table 1. Test conditions for acute and chronic BaP exposures in the Japanese medaka

Parameters	Conditions			
	Acute		Chronic	
	SC	BaP	SC	BaP
Exposure type	Water-borne	〃	〃	〃
Test organism	*Oryzias latipes*	〃	〃	〃
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
Exposure duration	48 hrs	〃	15 days	〃
pH	7.74±0.12	7.77±0.15	7.88±0.07	7.90±0.05
Dissolved oxygen (mg L^{-1})	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±0.1	25.03±0.03	24.11±0.29	24.47±0.20
Photoperiod	16 Light : 8 Dark	〃	〃	〃
Feeding regime	none	〃	Once a day	〃
Test water	Filterated seawater (0.22 μm cartridge filter)	〃	〃	〃

Value shown are mean±S.D., n=3.

biline DryStrip Reswelling Tray (Amersham Biosciences) with DeStreak Rehydration Solution (Amersham Biosciences) and 1.0% IPG buffer (Amersham Biosciences) for 16 hrs at room temperature. Rehydrated gels were transferred to an Ettan IPGphor System (Amersham Biosciences) and 100 μg of the protein samples were loaded into the samples cups. After the cups were overlaid with Immobiline DryStrip Cover Fluid (Amersham Biosciences), isoelectric focusing (IEF) was carried out at step and hold mode at 300 V for 1 hr, gradient mode at 1,000 V for 30 min, gradient mode at 5,000 V at 80 min, and step and hold mode at 5,000 V for 2 hrs, for a total of 7,000 V hrs. The completed IEF gels were equilibrated with sodium dodecyl sulphate (SDS) equilibration solution [6 M urea, 29.3% (v v^{-1}) glycerol (Amersham Biosciences), 2% (w v^{-1}) SDS, 75 mM Tris-HCl pH 8.8, and 0.002% (w v^{-1}) bromophenol blue] with DTT (100 mg mL^{-1}) and then idoacetamide (250 mg mL^{-1}) (Amersham Biosciences). Equilibrated gels were placed on the top of 10% SDS-polyacrylamide gels for electrophoresis and were sealed with 0.5% (w v^{-1}) agarose (Promega Bioscience, WI, USA). Following electrophoresis, the separated gels were incubated overnight in fixing solution (45% methanol and 5% phosphoric acid) and then stained with staining solution [0.1% (w v^{-1}) Comassie brilliant blue G250 (Bio-Rad Laboratories), 17% (w v^{-1}) ammonium sulfate, 3.6% phosphoric acid, and 34% methanol] for 1 day. Finally the gels were destained in washing solution (1% acetic acid and 15% methanol) for visualization. To analyze the stained spots, the gels were scanned using an ImageMaster Scanner (Amersham Biosciences) and then the spots were detected and analyzed using the ImageMaster 2D Platinum 6.0 software (GE healthcare, NJ, USA).

4. In-gel digestion of proteins

Protein spots of interest were excised and digested in-gel with sequencing grade, modified trypsin (Promega) as previously described (Bahk *et al.* 2004). In brief, each protein spot was excised from the gel, placed in a polypropylene tube, and washed 4 ~ 5 times (until the gel was clear) with 150 μL of 1 : 1 acetonitrile 25 mM ammonium bicarbonate^{-1}, pH 7.8. The gel slices were dried in a Speedvac concentrator and then rehydrated in 30 μL of 25 μM ammonium bicarbonate (pH 7.8) containing 20 ng of trypsin. After incubation at 37°C for 20 hrs, the liquid was transferred to a new tube. Tryptic peptides remaining in the gel matrix were extracted for 40 min at 30°C with 20 μL of 50% (v v^{-1}) aqueous acetonitrile containing 0.1% (v v^{-1}) formic acid. The combined supernatants were evaporated in a Speedvac concentrator and dissolved in 8 μL of 5% (v v^{-1}) aqueous acetonitrile solution containing 0.1% (v v^{-1}) formic acid prior to mass spectrometric analysis.

5. Identification of proteins by liquid chromatography-tandem mass spectrometry

The resulting tryptic peptides were separated and analyzed using reversed phase capillary high-performance liquid chromatograph directly coupled to a Finnigan LCQ ion trap tandem mass spectrometer (LC-MS/MS). Both a 0.1 × 20 mm trapping and a 0.075 × 130 mm resolving column were packed with Vydac 218 MS low trifluoroactic acid C18 beads (5 μm in size, 300 Å in pore size; Vydac, Hesperia, CA, USA) and placed in-line. The peptides were bound to the trapping column for 10 min with 5% (v v^{-1}) aqueous acetonitrile containing 0.1% (v v^{-1}) formic acid, and then the bound peptides were eluted with a 50 min gradient of 5-80% (v v^{-1}) acetonitrile containing 0.1% (v v^{-1}) formic acid at a flow rate of 0.2 μL min^{-1}. For tandem mass spectrometry, full mass scan range mode was m/z=450 ~ 2,000 Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with a relative collision energy of 55%.

The individual spectra from MS/MS were processed using the TurboSEQUEST software (Thermo Quest, CA, USA). The generated peak list files were used to query either the MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com). Modifications of methionine and cysteine, peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 0.8 Da, allowance of missed cleavage at 2, and charge states (+1, +2 and +3) were taken into account. Only significant hits as defined by MASCOT probability analysis were initially considered.

6. Statistical analysis

The difference for each spot, between control and BaP treatment groups, was determined based on its density. The statistical significance was analyzed with the student's *t*-test at $P<0.05$.

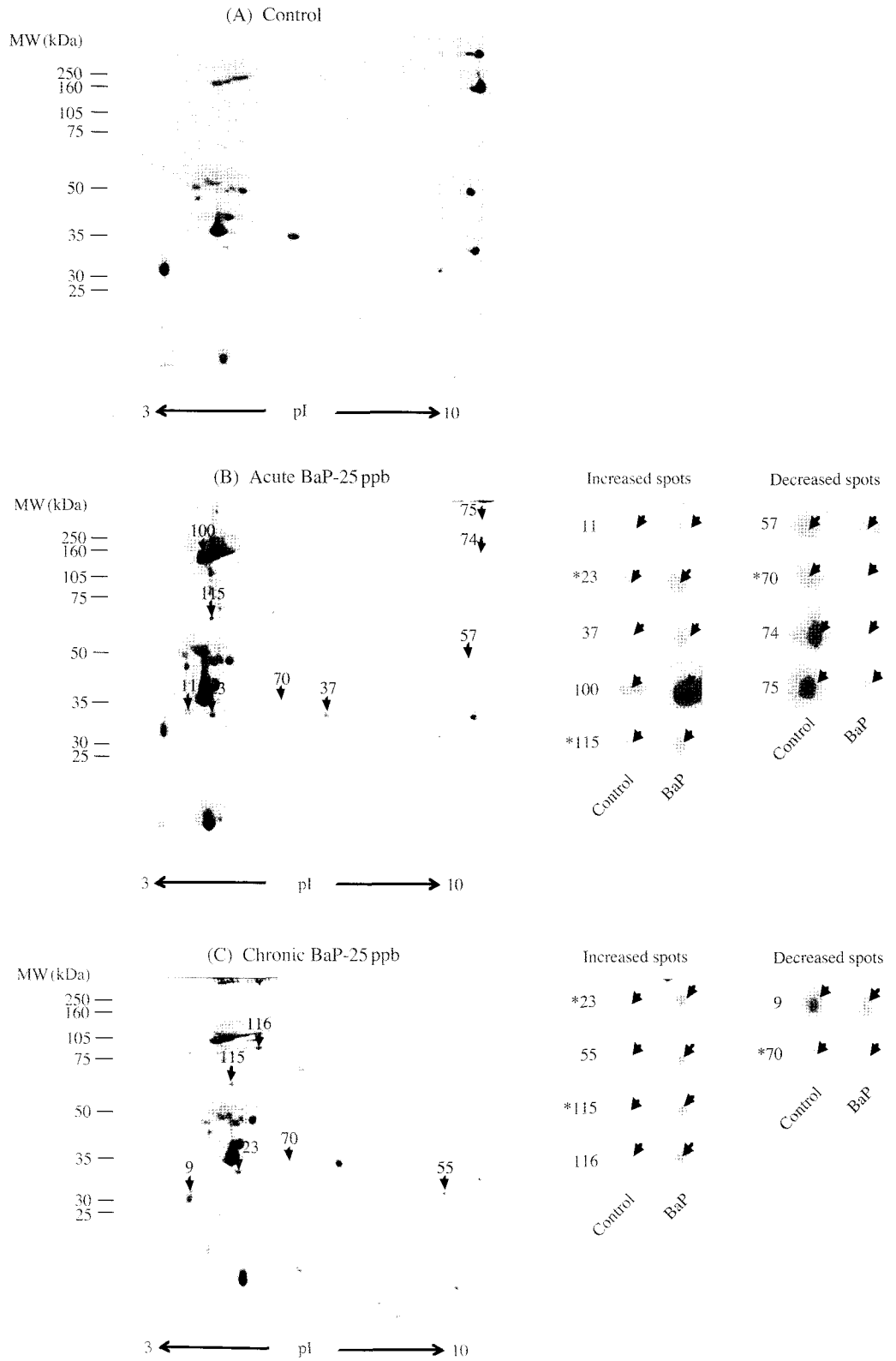

Fig. 1. Alteration of protein levels in the head of Japanese medaka (*O. latipes*) in response to acute (B) or chronic (C) exposure to BaP (25 μg L^{-1}). The fish were sacrificed at 48 hrs (acute) and 15 days (chronic) after waterborne exposure of BaP. As compared with the control group, 11 and 8 protein spots were determined to be statistically different following acute and chronic exposures of BaP, respectively. Statistical significance was determined with the student's *t*-test at $P < 0.05$. *, altered spots common to both acute and chronic groups.

Table 2. Identification of altered protein spots in response to acute and chronic BaP exposures in the Japanese medaka

Spot #	Protein	Mr(Da)/pI	Accession No.
23	Type I keratin S8 [*Oncorhynchus mykiss*]	47888/5.28	CAC45059
	Cytokeratin [*Stizostedion vitreum vitreum*]	31508/4.79	AAN01364
70	Muscle actin OIMA1 [*Oryzias latipes*]	41932/5.23	NP_001098276
	Alpha-actin [*Epinephelus coioides*]	36869/5.57	AAW29030
	Alpha-actin [*Oreochromis niloticus*]	34679/5.34	ABN58892
	Skeletal muscle actin [*Cyprinus carpio*]	41934/5.22	AAP74383
	Alpha actin [*Oreochromis niloticus*]	39265/5.28	ABN58896
	Alpha-cardiac actin [*Danio rerio*]	41969/5.29	AAF20165
	Actin, cytoplasmic 3 (Beta-actin C) [*Takifugu rubripes*]	41756/5.30	P53486
	Unnamed protein product [*Tetraodon nigroviridis*]	41159/5.15	CAG12586
	Alpha actin [*Oreochromis niloticus*]	39218/5.47	ABN58888
	Beta cytoplasmic actin [*Scophthalmus maximus*]	17475/5.08	ABJ98691
	Beta-actin [*Siniperca chuatsi*]	19667/5.30	AAW77932
	Actin, alpha anomalous [*Takifugu rubripes*]	41952/5.11	P53483
115	Beta actin [*Morone saxatilis*]	31500/5.06	AAA53024

RESULTS AND DISCUSSION

The biological effects of BaP on the head of Japanese medaka were investigated by the characterization of altered proteins using proteomic and bioinformatic analyses. The alteration of protein levels in the head of the medaka was observed after acute or chronic exposure to BaP (25 $\mu g\ L^{-1}$) for 48 hrs and 15 days, respectively. The data demonstrated that approximately 112 protein spots were detected on the 2-DE gels (data not shown). Among them, there were 9 and 6 protein spots that were determined to be statistically different in response to acute and chronic BaP exposures, respectively (Fig. 1). In the acute exposure group, 5 spots (11, 23, 37, 100, 115) were upregulated and 4 spots (57, 70, 74, 75) were downregulated. In the chronic exposure group, 4 spots (23, 55, 115, 116) were upregulated and 2 spots (9, 70) were downregulated. Three spots (23, 70, 115) that were common to both the acute and chronic exposure groups were identified and summarized in Table 2.

The results showed that the altered proteins were found to be associated with structural functions, including the actin and keratin families of proteins. These data suggest that the acute and chronic exposures to BaP could adversely affect the expression of proteins responsible for the formation of the cytoskeleton in aquatic organisms.

The cytoskeleton plays a significant role in structural support, intracellular transport, and cell motility, and it also helps to organize the contents of cells (Lodish *et al.* 2003; Pollard *et al.* 2003). In order to adapt or survive environmental pollution, cells need the coordinated regulation of highly conserved cytoskeleton-associated proteins. Actin family proteins are the most abundant proteins in most eukaryotic cells, which form a static structure consisting of a network of filaments that can readily affect cell morphology (Pollard *et al.* 2000; Fehrenbacher *et al.* 2003; Engqvist-Goldstein and Drubin 2003). Similar to actin, keratin family proteins are expressed in epithelia and are easily exposed to physical and biochemical stresses, and their structural integrity is essential for maintaining the strength and rigidity to the entire epithelium (Coulombre *et al.* 2002). Deficiencies in the cytoskeleton and loss of resistance to various stimuli, including exposure to toxic chemicals, may cause pathological conditions in marine organisms. This is supported by the observation that cells and tissues with missing or abnormal intermediate filaments exhibit structural or physical defects as well as more complex pathophysiological phenomena (Galou *et al.* 1997; Pekny and Lane 2007). In summary, these data suggest that acute or chronic exposure to BaP has an affect on tissue morphology in the head of Japanese medaka, and the information obtained from the present study could establish a new criterion for risk and hazard assessment of marine environmental contamination.

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