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Evaluation of biomarker potential of cytochrome P450 1A (*CYP1A*) gene in the marine medaka, *Oryzias melastigma* exposed to water-accommodated fractions (WAFs) of Iranian crude oil

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ABSTRACT

CYP1A is involved in the metabolism of diverse chemicals, including polycyclic aromatic hydrocarbons and alkylated-PAHs, as a first line of detoxification mechanism. First, we identified and characterized the *CYP1A* gene from the marine medaka, *Oryzias melastigma*. *O. melastigma* CYP1A (Om-CYP1A) showed a high similarity of motifs/domains compared to those of vertebrates in their amino acid sequences. To check whether the *Om-CYP1A* would be inducible, we tested two strong CYP1A inducers, β-naphthoflavone (β-NF) and benzo[α]pyrene (B[α]P), and observed concentration-dependent transient expression on transcripts of *Om-CYP1A* for 96 h over a wide range of concentrations. *Om-CYP1A* mRNA level was significantly increased in exposure to different concentrations of β-NF and B[α]P, and its expression was highly transcribed within 12 h upon the exposure to low concentrations of both chemicals. Inducible transcript profiles revealed that *Om-CYP1A* would be associated with the toxicant metabolism via AhREs/DREs/XREs in its promoter region. To uncover the effects of the water-accommodated fraction (WAF) of crude oil on transcripts of *Om-CYP1A*, we measured mRNA expression of *Om-CYP1A* towards different concentrations of WAF for 24 h. As a result, WAF exposure significantly increased *Om-CYP1A* transcripts at all concentrations as well as during time-course experiments for 96 h. In this paper, we demonstrated that WAF would trigger up-regulation of the *CYP1A* gene that would be associated with the initiation of the cellular defense systems. This finding provides a better understanding of the molecular mechanism of cellular protection particularly that involved in the WAF-mediated cellular response in *O. melastigma*.

1. Introduction

The cytochrome P450 superfamily (CYP) is known as a large group of enzymes that are involved to catalyze the oxidation of diverse organic substances in a wide range of different metabolic reactions including drug metabolism and bioactivation (Guengerich, 2008). Among CYPs, the *CYP1A* (encodes cytochrome P450, family 1, subfamily A in human) gene is one of the major members of the CYP1 family, and it has several important roles in phase I xenobiotic/drug metabolism as well as in the metabolic activation of aromatic hydrocarbons. The transcriptional activation of the *CYP1A* gene is mediated through a cytosolic receptor, aryl hydrocarbon receptor (AhR), and its translocation as a dimer formation with an aryl hydrocarbon receptor nuclear translocator (Arnt) to the nucleus (Hankinson, 1995). This regulation pathway appears to be similar in fish and mammals. Consequently, numerous studies have investigated the expression pattern of CYP1A in diverse animals, which are exposed to xenobiotics and environmental pollutants, and its expression has further been reported in diverse fish species using molecular techniques such as RT-PCR, real-time PCR, activity analysis of the enzyme, Western blotting, and immunohistochemistry (Sarasquete and Segner, 2000).

In fish, *CYP1* gene families are biomarkers for several xenobiotics and AhR agonist exposure. In fact, a β-naphthoflavone (β-NF), a model AhR agonist and CYP1A inducer, induced hepatic *CYP1A* in several fish species (Arukwe, 2002; Bard et al., 2002; Meyer et al., 2002; Ryu et al., 2004;

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Quirós et al., 2007; Kim et al., 2008; Søfteland et al., 2010). Other chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), B[α]P, and some polychlorinated biphenyl (PCB) have been recognized as strong AhR ligands as well as *CYP1A* inducers in fish species. In addition, several recent reports suggested that *CYP1A* induction would provide a potential biomarker for environment monitoring in response to heavy oil exposure or oil spills. Arukwe et al. (2008) suggested that increased mRNA expression of *CYP1A* gene might be a sensitive biomarker to the water-accommodated fraction (WAF) exposure in zebrafish. Similar acute induction patterns of the *CYP1A* transcript were observed in crude oil-exposed rainbow trout (Hook et al., 2010). Weathered oil treatment also significantly induced *CYP1A* transcription in European seabass (Della Torre et al., 2012). Sundt et al. (2012) reported that hepatic *CYP1A* gene expression significantly up-regulated in Atlantic cod after exposure to authentic produced water (PW) from a North Sea oil field. Thus, analysis of the mRNA/protein expression profile of *CYP1A* gene within fish is still considered as a reliable tool in mechanistic studies of oil pollution.

The marine medaka, *Oryzias melastigma* is a potential marine model fish that could be used in diverse fields of fish biology and ecotoxicology (Kong et al., 2008). Many experimental advantages of marine medaka are similar to the freshwater Japanese ricefish *O. latipes*. They are small and easy to culture, and can breed in seawater. They also exhibit uniform growth and have several promising features such as short generation time, hardiness to extreme environmental changes, susceptibility to chemicals, and adaptation to a wide range of salinities. In addition to these factors, anatomy, biology, and nutritional requirements are similar to those of the freshwater *O. latipes*. Even though partial cDNA sequence of the *CYP1A* gene was recently cloned in this species and mRNA expression was tested for perfluorooctane sulfonate (PFOS) exposure (Fang et al., 2012), vast majorities for gene information and characterization of gene expression still remain unclear. In this paper, we report on molecular cloning, sequence analysis of marine medaka *CYP1A* gene, and expression of *Om-CYP1A* mRNAs in early developmental stages and different tissues. Also, we characterize modulated patterns of *Om-CYP1A* gene under exposure to water-accommodated fractions (WAFs) in order to provide a better understanding of the potential role of this gene for biomonitoring studies of oil contaminated marine areas.

2. Materials and methods

2.1. Fish

The marine medaka *O. melastigma* (provided from Prof. Doris W.T. Au's lab, City University of Hong Kong in 2009) were reared and maintained at the aquarium of the Department of Chemistry, College of Natural Sciences, Hanyang University, Seoul in South Korea. The fish were maintained at 25 °C at 12 h/12 h light/darkness and 12 psu (practical salinity unit) salinity. The automated water changing system was set for constant flow-through, and the water quality (pH, salinity, and temperature) was recorded using various instruments. This system could also provide photoperiodicity (12L: 12D) automatically a day. They were fed on a diet of *Artemia salina* (<24 h after hatching) once a day until satiation.

2.2. Total RNA extraction

Fish were anesthetized on ice and killed by decapitation. Liver tissues were quickly removed under sterile conditions and homogenized in three volumes of TRIZOL® (Invitrogen, Paisley, Scotland, UK) with a glass tissue grinder. Total RNA was extracted according to the manufacturer's instructions and stored at −80 °C until use. DNA digestion was performed using DNase I (Sigma, St. Louis, Mo). Total RNA was quantified by absorption of light at 230, 260, and 280 nm (A230/260, A260/280) using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience). To check genomic DNA contamination, we loaded total RNA in a 1% agarose gel which contained ethidium bromide (EtBr) and visualized on UV transilluminator (Wealtec Corp., NV, USA). Subsequently, we loaded total RNA in a 1% formaldehyde/agarose gel with EtBr staining to verify for total RNA quality, and checked the 18/28S ribosomal RNA integrity. After RNA quality was determined, single-strand cDNA was synthesized from 2 μg of total RNA from each sample using oligo $(dT)_{20}$ primer for reverse transcription in 20 μL reactions (SuperScript ™ III RT kit, Invitrogen, Carlsbad, CA).

2.3. cDNA cloning and promoter region analysis of Om-CYP1A

The partial genomic DNA sequence of *Om-CYP1A* was obtained by GS FLX-Titanium genomic DNA sequencer (Roche Diagnostics, Mannheim, Germany). Subsequently, *Om-CYP1A* gene was subjected to 5′-Rapid Amplification of cDNA Ends (5′-RACE) to confirm the full-length cDNA sequence according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and to check the exon/intron boundary. A series of RACE was performed with specific primers (Table 1) under the following conditions: 94 °C/4 min; 40 cycles of 98 °C/25 s, 55 °C/30 s, 72 °C/60 s; and 72 °C/10 min. The final PCR products were isolated from 1% agarose/TBE gel, cloned into pCR2.1 TA vectors (Invitrogen), and sequenced with an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea). The sequence of the promoter region of *Om-CYP1A* was analyzed with Genetyx version 7.0 software.

2.4. Sequence analysis

The sequence of *Om-CYP1A* cDNA and the amino acid residues were refined using Genetyx software (ver. 7.0.3). The amino acid sequences were aligned with those of other species retrieved from GenBank database using Clustal X (ver. 1.83) and GENEDOC software (ver. 2.6). The conserved domains were searched using the NCBI Conserved Domain Database (CDD, ver. 2.30) (http://www.ncbi.nlm.nih.gov/Structure/cdd).

2.5. Real-time RT-PCR

For real-time RT-PCR (RT-PCR), 1 μL of cDNAs was used with other ingredients including 0.2 μM primer (real-time RT-F/R and *18S rRNA* RT-F/R; Table 1). Reaction conditions were as follows: 94 °C/4 min; 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and 72 °C/10 min. SYBR® Green (Molecular Probe Inc., Invitrogen) was used to detect specific real-time RT-PCR products. To confirm the amplification of a specific product, cycles were continued to check the melting curve under the following conditions; 95 °C/1 min, 55 °C/1 min, and 80 cycles of 55 °C/10 s with 0.5 °C increase per cycle. To determine the amplicon identity, the PCR product was cloned into pCR2.1 TA vector (Invitrogen, Carlsbad, CA), and sequenced with an ABI 3700 DNA analyzer (Bionics Co., Seoul, South Korea). Optimized conditions were transferred according to the following CFX96™ real-time PCR system protocol (Bio-Rad). The *O. melastigma 18S rRNA* gene was used as a reference gene. All the data of triplicate experiments were expressed

Table 1
Primers used in the study.

Gene	Oligo name	Sequence (5′→3′)	Remarks	Amplicon (bp) (efficiency)
Om-CYP1A	F1	ATACTGCCATTCATCGGTCCTC	Cloning	
	R1	GCGCTTGTGCTTCATTGTGAG	5′-RACE	
	5GSP1	GCCACAGACACAACAATGTATC		
	5GSP2	CTCCAACAGATTCCCAATGATG		
	RT-F	TCGTCGTTCTAAGTGGCAATGAAAC	Real-time PCR	138 (98.18%)
	RT-R	AGAAAGAGCGCAATGCACTGTAGG	amplification	
Om-18S rRNA	RT-F	CCTGCGGCTTAATTTGACCC	Real-time PCR	106 (99.23%)
	RT-R	GACAAATCGCTCCACCAAC	amplification	