J. of Aquaculture
Vol. 16(2) : 99-103, 2003

한국양식학회지
Journal of Aquaculture
©Korean Aquaculture Society

Hepatic Response in Cytochrome P450 and De-alkylase Activity of Olive Flounder (*Paralichthys olivaceus*) Exposed to Water-borne Phenanthrene

Jung-Hoon Jee, Dae-Kuk Park and Ju-Chan Kang*
Department of Aquatic Life Medicine, Pukyong National University, Busan 608-737, Korea

Olive flounder (*Paralichthys olivaceus*) was exposed to water-borne phenanthrene for 4 weeks. After the exposure for 2-weeks, hepatic cytochrome P450 contents were significantly elevated. Induction of hepatic ethoxy resorufin-*O*-deethylase (EROD) activity was significantly increased in flounders treated with 1.0 and 2.0 μM phenanthrene, compared to untreated group and 0.5 μM treated group. However, there were no significant changes in pentoxyresorufin-*O*-deethylase (PROD) activity in hepatic microsome of all the phenanthrene-treated groups, compared to the untreated group. Phenanthrene has the potential to induce cytochrome P450 and EROD enzyme of the olive flounder.

Keywords: *Paralichthys olivaceus*, Phenanthrene, CYP450, EROD, PROD

Introduction

Approximately 230,000 tons of total Polycyclic Aromatic Compounds (PAHs) enter aquatic environments yearly, worldwide (Eisler, 1987). Hence the PAHs are widespread in the environment. Because of their carcinogenic and mutagenic properties (Lehr and Jerina, 1977), they have been intensively studied in different aquatic systems (McElroy et al., 1989). They are known to induce members of the cytochrome P-450 enzyme family, some of which are responsible for the bio-activation of organophosphate insecticides to their much more potent oxon analogues. A high incidence of liver tumors has been reported in several species of bottom-feeding fish collected from areas contaminated with these chemicals (Baumann et al., 1982). With three aromatic rings, phenanthrene accounts for 0.3-3% of the total composition of crude oil (NASA, 1985), and is a PAH that is ubiquitously present in the environment, as a product of incomplete combustion of fossil fuels and wood. It has been identified in ambient air, surface and drinking water, and in food (U.S. EPA, 1988). It is known that many Korea estuaries are contaminated with a wide range of pollutants including PAHs (Moon et al., 2001).

Oil-water dispersion was very toxic to fish, as oil droplets make contact with the gills and directly damage the respiratory epithelium (Engelhardt et al., 1981). Similarly, the diesel water-soluble fraction (DWSF) produced alterations in the respiratory rate of fish (Anderson et al., 1974), hematocrit and oxygen consumption increases (Davison et al., 1992), reduced rates of growth, survival, and reproduction (Ernst et al., 1977), as well as behavioral stress responses (Davison et al., 1992).

Despite previous investigations carried out on this subject, some toxicological response levels in fish remain poorly understood, revealing in particular, the lack of data concerning the stress mechanism, as well as biotransformation and genotoxic responses. Biotransformation studies in fish revealed that exposure to PAHs induces cytochrome P450-dependent enzymatic activities such as ethoxyresorufin *O*-deethylation (EROD). Therefore, the determination of EROD activity has been adopted as a valuable indicator, for the detection of PAHs at presumably toxic levels (van Veld et al., 1990; Addison et al., 1994). Although a large body of literature is available on the toxicity of PAHs, primarily benzo[*a*]pyrene, toxicity data for phenanthrene are very limited.

Aquatic environments have been used for decades as a major repository of anthropogenic wastes. Among the types of pollutants typically attributable to human activities, petroleum products are one of the most several laboratory experiments, with different organisms, have been carried out on the toxicity of water-soluble fraction, oil-water dispersions. However, only a few experiments concern fish. In the present

*Corresponding author: jckang@pknu.ac.kr

study, an attempt is made to define cytochrome P450 mixed-function mono-oxygenase (MFO) system of olive flounder, *Paralichthys olivaceus*, exposed to water-borne phenanthrene.

Material and Methods

Test organisms

Olive flounder (*Paralichthys olivaceus*) of 51 ± 4.3 g body weight were obtained from a commercial dealer and transferred to a maintenance room, where temperature was kept constantly at 20 ± 1 °C. They were acclimatized to laboratory conditions for 2 weeks in 300 L tanks prior to the experiments. Test chambers (glass aquaria, 50 L capacity each) were filled with 40 L of sea water each. Supplemental aeration was provided to maintain the dissolved oxygen concentrations near saturation. Water characteristics, measured by the method described in APHA(1985), were as follows: pH: 8.03 ± 0.4; temperature: 20 ± 1 °C; salinity: 31.8 ± 0.7‰; and dissolved oxygen: 7.4-7.9 mg/L.

Exposure condition

Phenanthrene was initially dissolved in ethanol (Sigma Chemical, St. Louis, MO) to obtain initial stock solution at 35.64 mg/ml. Fifteen minutes prior to introduction of fish to 50 L aquaria, solutions of phenanthrene (>96% purity, Sigma Chemical, St. Louis, MO) and working solution were mixed with water to attain the desired nominal concentration of 0.5, 1.0, 2.0 μM. The working solutions were stirred 816 h prior to use. Final ethanol concentration in any of the treatments did not exceed 0.1%. In each test chamber, a group of 7 individuals was exposed for a period of 2 and 4 weeks under semi-static conditions. The phenanthrene dose was renewed every second day, along with the seawater. The control received the same amount of ethanol.

Sampling

At the end of experimental period, fish were anesthetized with 3-amino benzoic acid ethyl ester methane sulfonate (Sigma Chemical, St. Louis, MO) and hepatic microsome were prepared by homogenizing in ice cold buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM dithiothrietal, 150 nM NaCl) with several passes of a teflon pestle (099C K4424, Glas-Col, USA). The homogenate was centrifuged (9,000 g for 20 min, MIKRO 22R, Hettich, Germany) at 4 °C, supernatant was collected, and ultracentrifuged at 100,500 g for 60 min using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Italy) at 4 °C to obtain a microsomal pellet. The pellet was resuspended in 0.1 M phosphate buffer (1 mM Na_2EDTA, 1 mM dithiothreitol (DTT), 20% glycerol, pH 7.4).

Cytochrome P450 assay

Cytochrome P450 content was determined by carbon mono-oxide-difference spectra of dithionite-reduced microsomes using an extinction coefficient of 91 mM^{-1} cm^{-1} for A_{450}-A_{490} (Omura and Sato, 1964).

Hepatic de-alkylase assays

Hepatic EROD activity was determined using a modification of the multiwell plate method of Kennedy and Jones (1994). The following reagent concentrations were used in the EROD reaction mixture: 7-ethoxyresorufin, 1.7 μM (Sigma, USA); NADPH, 0.5 mM (Boehringer Mannheim, Germany); $MgSO_4$, 17 mM; and HEPES, 0.1 M, pH 7.8 M (Sigma, USA). EROD activity was determined in 48-well culture plates (Costar, Cambridge, MA) using a plate reading flourometer (BF10001, Packard bioscience Co.). Excitation and emission filters were set at 530 nm and 590 nm, respectively. Activity was determined at 25 °C using 25 μl post-mitochondrial supernatant in the reaction mixture. The penthoxyresorufin *O*-deethylation (PROD) activity was assayed in the same system, as EROD. Enzyme activities were calculated using the fluorescence increase caused by the addition of resorufin. Protein concentration was determined using Bradford (1976), with a bovine serum albumin (Sigma, USA) as standard.

Statistical analysis

Statistical analyses were performed with the aid of the SPSS/PC+statistical package. Significant differences between means were determined using one-way ANOVA and the Duncan's test for multiple comparisons (Duncan, 1955). Significance level was established at $P<0.05$.

Results

The effect of water-borne phenanthrene exposure to 4 weeks on P450 in liver of flounders is depicted in Fig. 1. Liver cytochrome P450 contents in control and 0.5 μM were stable during the test period. However, hepatic cytochrome P450 contents were significantly increased after 2 week exposure to phenanthrene. Compared with those of the control group, the values were about two times higher for hepatic cytochrome P450 contents in flounders exposed to 2.0 μM phenanthrene at 4

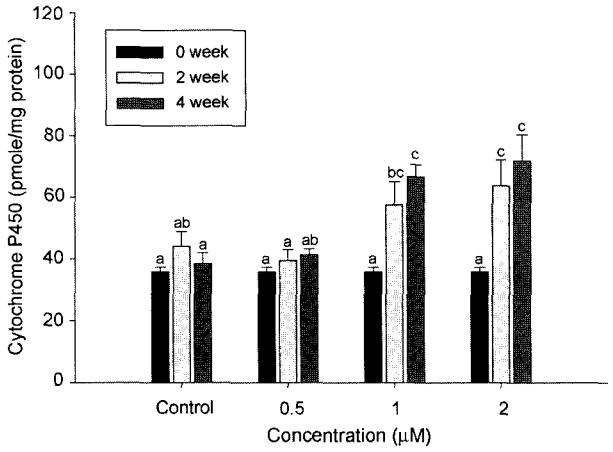

Fig. 1. Hepatic cytochrome P450 contents of flounders exposed to water-borne phenanthrene for 4 weeks. Data are presented as mean±S.E. Values with different superscripts are significantly different (P<0.05) as determined by Duncan's multiple comparison.

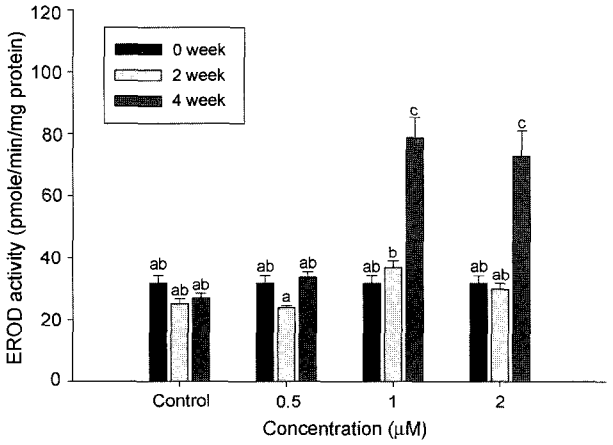

Fig. 2. Hepatic EROD activity of flounders exposed to water-borne phenanthrene for 4 weeks. Data are presented as mean±S.E. Values with different superscripts are significantly different (P<0.05), as determined by Duncan's multiple comparison.

weeks (P>0.05).

Fig. 2 shows the effect of phenanthrene on EROD activity in liver of flounders. Hepatic EROD activity was increased insignificantly in all the treated groups at the sampling period of 2 weeks and thereafter the increase was observed in groups exposed to 1.0 μM and 2.0 μM phenanthrene. Significant increment was confirmed at the concentrations of 1.0 and 2.0 μM, in which the increases in EROD activity were 2.5 (P<0.05) and 2.3 (P<0.05) fold of the control, respectively.

There was an incline in PROD in the hepatic microsome in the phenanthrene-treated groups compared to that of control group. However, there were no significant changes in all the exposed groups compared to controls (Fig. 3, P>0.05).

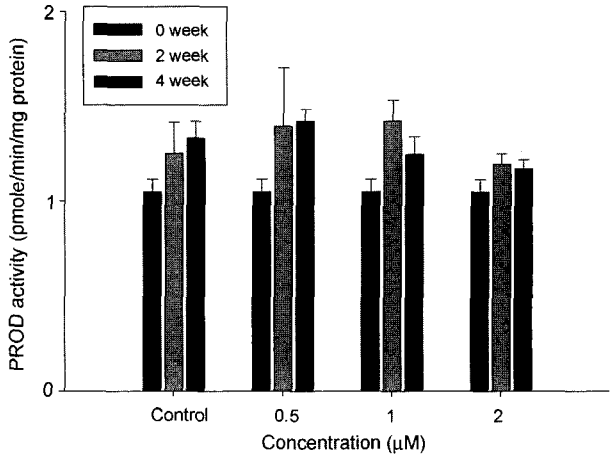

Fig. 3. Hepatic PROD activity of flounders exposed to water-borne phenanthrene for 4 weeks. Data are presented as mean±S.E.

Discussion

The presented results clearly indicates that an exposure of the flounder to phenanthrene induced hepatic cytochrome P450 and de-alkylase activity. The duration and intensity of action of xenobiotics within a biological system are determined by the rate of their biotransformation to pharmacologically active or inactive metabolites. Cytochrome P450, a heme protein, is a heterogeneous system of microsomal enzymes responsible for the oxidative biotransformation of many chemicals (including drugs) to polar metabolites, thereby facilitating the pharmacological inactivation of these chemicals and their elimination from the body (Lu and West, 1980; Wrighton and Stevens, 1992). Cytochrome P450 occurs in multiple forms, and the composition of these isoenzymes, as well as their relative concentrations in tissues, are influenced by treatment with different chemicals (Lu and West, 1980).

In this study, there was a significant increase hepatic cytochrome P450 level in phenanthrene exposure groups (Fig. 1). The value of liver cytochrome P450 concentration of 2.0 μM exposure group was significantly different from those of control for the exposure duration of either 2 or 4 weeks (P<0.01). At the end of the experiment, the cytochrome P450 content of 2.0 μM exposure group was higher and exhibited an almost two-fold compared to the values of control group. Thus, a pronounced increase occurred in hepatic cytochrome P450 induction in the presence of phenanthrene, which confirms the previous statement that cytochrome P450 induction in the flounder can be a reliable indicator of contamination of the aquatic environment by compounds known to induce CYP1A, such as many xenobiotics (Collier et al., 1995).

As the bottom dwelling species, the flounder is apparently suitable for monitoring pollution of sediments and its effect in coastal area. The European flounder (*Platichthys flesus* L.) is thus often strongly inflicted by liver cancer because of its close contact to the sediments loaded with hepatotoxic compounds (Malins et al., 1984, 1985; Myers et al., 1990). Flounder is a stationary, benthic organism and one of the most important fish in Korean aquaculture.

Ethoxyresorufin O-deethylase (EROD) activity in fish liver is increasingly used to indicate the presence or the effects of certain organic contaminants. This is because laboratory studies have been established a strong causal link between exposure of the fish to contaminants and expression of cytochrome P-4501A1 and its EROD activity (Haasch et al., 1989; Skaare et al., 1991). Although, monitoring of cytochrome P-4501A1 and its EROD activity have been widely used as a biomarker of aquatic pollution, the effect of phenanthrene on these parameters has not yet been established. Our results have shown that following water-borne exposure of phenanthrene (2.0 μM), there was significant induction of hepatic EROD at the 4th week ($P<0.05$).

In this study, no significant difference between phenanthrene-treated and control groups in hepatic PROD activity was observed. The PROD activity is a catalytic probe for determining the induction response of CYP2B class isozyme in mammals. In fish P450 system, the phenobarbital (PB) type inductive response appears to be completely absent (Goksøyr and Förlin, 1992). It has been established that CYP2B genes are present and expressed in fish, but that they are non-responsive to PB-type compounds (Stegeman et al., 1990). In a study by Brown (1992), a large series of gas chromatograms of the PCB residues in 32 species of teleost fish was investigated. CYP2B-like alteration patterns were observed in only 4 species. In the present study, insignificant PROD induction was observed in the phenanthrene-treat flounder. Therefore, phenanthrene seems not to induce hepatic CYP2B class isozyme.

In conclusion, water-borne phenanthrene significantly affected the content of cytochrome P450 and EROD activity. The level of depression, induced by chronic exposure to phenanthrene, can be used as a biomarker to predict coastal and estuarine risk assessment.

Acknowledgements

This work was supported by a grant No. R05-2001-000-00774-0 from Korea Science & Engineering Foundation.

Reference

Addison, R. F., D. E. Willis and M. E. Zinck, 1994. Liver microsomal mono-oxygenase induction in winter flounder (*Pseudopleuronectes americanus*) from a gradient of sediment PAH concentrations at Sydney Harbour, Nova Scotia. Mar. Environ. Res., **37**: 283–296.

Anderson, J. W., J. M. Neff, B. A. Cox, H. E. Tatem and G. M. Hightower, 1974. Characteristics of dispersions and water soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar. Biol., **27**: 75–88.

APHA (American Public Health Association), 1985. American water works association, and water pollution control federation. Standard methods for the examination of water and waste water. 16th edition. APHA, Washington, D.C., 1268 pp.

Baumann, P. C., W. D. Smith and M. Ribbick, 1982. In Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry. Battelle Press, Columbus, OH. pp. 93–102.

Bradford, M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**: 248–254.

Brown, J. F., 1992. Metabolic alteration of PCB residues in aquatic fauna: distributions of cytochrome P4501A1- and P4502B-like activities. Mar. Environ. Res., **34**: 261–266.

Collier, T. K., B. F. Anulacion, J. E. Stein, A. Goksoyr and U. Varanasi, 1995. A field evaluation of cytochrome P4501A as a biomarker of contaminant exposure in three species of flatfish. Environ. Toxicol. Chem., **14**: 143–152.

Davison, W., C. E. Franklin, J. C. McKenzie and M. R. Dougan, 1992. The effects of acute exposure to the water soluble fraction of diesel fuel oil on survival and metabolic rate of an Antarctic fish (*Pagothenia borchgrevinki*). Comp. Biochem. Physiol., **102C**: 185–188.

Duncan, D. B., 1995. Multiple-range and multiple F tests. Biometrics, **11**: 1–42.

Eisler, R., 1987. Polycyclic aromatic hydrocarbons hazards to fish, wildlife, and invertebrates: a synoptic review. Fish. Wild. serv. Biol. Rep., **104C**: 67–70.

Engelhardt, F. R., M. P. Wong and M. E. Duey, 1981. Hydromineral balance and gill morphology in rainbow trout *Salmo gairdneri*, acclimated to fresh and sea water, as affected by petroleum exposure. Aquat. Toxicol., **1**: 175–186.

Ernst, V. V., J. M. Neff and J. W. Anderson, 1977. The effects of the water-soluble fractions of No. 2 fuel oil on the early development of the estuarine fish, *Fundulus grandis* Baird and Girard. Environ. Pollut., **14**: 25–35.

Goksøyr, A. and L. Förlin, 1992. The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol., **22**: 287–312.

Haasch, M. L., P. J. Wejksnora, J. J. Stegeman and J. J. Lech, 1989. Cloned rainbow trout liver P 450 complementary DNA as a potential environmental monitor. Toxicol. Appl. Pharmacol., **98**: 362–368.

Kennedy, S. W. and S. P. Jones, 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein con-

centration with a fluorescence plate reader. Anal. Biochem., **222**: 217–223.

Lehr, R. E. and D. M. Jerina, 1977. Metabolic activation of polycyclic hydrocarbons. Arch. Toxicol., **39**: 1–6.

Lu, A. Y. H. and S. B. West, 1980. Multiplicity of mammalian microsomal cytochrome P-450. Pharmacological Reviews, **31**: 277–295.

Malins, D. C., B. B. McCain, W. D. Brown, S. L. Chan, M. S. Myers, J. T. Landahl, P. G. Prohaska, A. J. Friedman, L. D. Rhodes, D. G. Burrows, W. D. Gronlund and H. O. Hodgins, 1984. Chemical pollutants in sediments and diseases in bottom-dwelling fish in Pudged Sound, Washington. Environ. Sci. Technol., **18**: 705–713.

Malins, D. C., M. M. Krahn, M. S. Myers, L. Rhodes, W. D. Brown, C. A. Krone, B. B. McCain and S. Chan, 1985. Toxic chemical in sediments and biota from a creosote-polluted harbour: relationship with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). Carcinogenesis, **6**: 1463–1469.

McElroy, A. E., J. W. Farrington and J. M. Teal, 1989. Bioavailability of polycyclic aromatic hydrocarbons in the aquatic environment. (*in*) Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment, (ed). U. Varanasi CRC Press, Boca Raton, FL., pp. 1–40.

Moon, H. B., H. G. Choi, S. S. Kim, S. R. Jeong, P. Y. Lee and G. Ok, 2001. Monitoring of polycyclic aromatic hydrocarbon in sediments and organsisms from Korean coast.. J. Fish. Sci. Tech., **4**: 219–228.

Myers, M. S., J. T. Landahl, M. M. Krahn, L. L. Johnson and B. B. McCain, 1990. Overview of studies on liver carcinogenesis in English sole from Pudget Sound; evidence for a xenobiotic chemical etiology. Pathology and epizootiology. Sci. Total. Environ., **94**: 33–50.

NASA (National Academy of Sciences), 1985. Oil in the sea-inputs, fates and effects. pp. 19–20.

Omura, T. and R. Sato, 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. J. Biol. Chem., **239**: 2379–2385.

Skaare, J. U., E. G. Jensen, A. Goksoeyr, E. Egaas, 1991. Response of xenobiotic metabolizing enzymes of rainbow trout (*Oncorhynchus mykiss*) to the mono-ortho substituted polychlorinated PCB congener 2, 3', 4, 4', 5-pentachlorobiphenyl, PCB-118, detected by enzyme activities and immunochemical methods. Arch. Environ. Contam. Toxicol., **20**: 349–352.

Stegeman, J. J., B. R. Woodlin and R. M. Smolowitz, 1990. Structure, function and regulation of cytochrome P450 forms in fish. Biochem. Soc. Trans., **18**: 19–21.

U.S. EPA (U.S. Environmental Protection Agency) 1988. Drinking Water Criteria Document for Polycyclic Aromatic Hydrocarbons (PAHs). Prepared by the Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, U.S. Environmental Protection Agency, Cincinnati, pp. 7–8.

van Veld, P. A., D. J. Westbrook, B. R. Woodin, R. C. Hale, C. L. Smith, R. J. Hugget and J. J. Stegeman, 1990. Induced cytochrome P-450 in intestine and liver of spot (*Leiostomus xanthurus*) from a polycyclic aromatic hydrocarbon contaminated environment. Aquat. Toxicol., **17**: 119–132.

Wrighton, S. A. and J. C. Stevens, 1992. The human hepatic cytochrome P-450 involved in drug metabolism. Crit. Rev. Toxicol., **22**: 1–22.

Manuscript Received: February 27, 2003
Revision Accepted: April 4, 2003
Responsible Editorial Member: Myung-Joo Oh