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Effect of Phenanthrene on Gill Biotransformation in Olive Flounder (*Paralichthys olivaceus*)

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The potential of phenanthrene to induce xenobiotic responses was investigated. Olive flounder (*Paralichthys olivaceus*) was exposed to different levels of phenanthrene (0.5, 1 and 2 μM) for 4 weeks. Gill CYP450 content and EROD (ethoxyresorufin *O*-deethylation) activity were found to be significant in the flounders treated with the higher concentration of phenanthrene (>1.0 μM), however, there were no significant changes in gill PROD (penthoxyresorufin O-deethylation) activity in all treated groups compared to the controls. This study demonstrated that phenanthrene has potential to induce gill cytochrome P450 and EROD enzyme in olive flounder.

Key words: CYP450, EROD, Gill, *Paralichthys olivaceus*, Phenanthrene

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are becoming ubiquitous in estuarine and coastal areas under human influence. PAHs commonly detected in marine sediment, water and organism (Wade et al., 1988; Kennicutt et al., 1988; McElroy et al., 1989).

Phenanthrene, also known as phenanthrin, is one of PAH with three aromatic rings. It has a chemical formula of $C_{14}H_{10}$, a molecular weight of 178.22, and exists as a colorless crystalline solid (U.S. EPA, 1987). Phenanthrene is a ubiquitous contaminant in the environment and a suspect carcinogen (Law et al., 1997). Phenanthrene occurs in fossil fuels and is present in products of incomplete combustion. Some of the known sources of phenanthrene in the environment are vehicular emissions, coal and oil burning, wood combustion, coke plants, aluminum plants, iron and steel works, foundries, municipal incinerators, synfuel plants, and oil shale plants (U.S. EPA, 1987).

Polycyclic aromatic compounds are widespread in the environment. Due to their carcinogenic and mutagenic properties (Lehr and Jerina, 1977), these compounds have been intensively studied in the various compartments of the environment (McElroy et al., 1989). Despite previous investigations carried out on this subject, some toxicological responses of fish remain poorly understood. In particular, there are few studies have conducted on the stress mechanism, as well as biotransformation and genotoxic responses. Biotransformation studies in fish revealed that PAHs exposure induces cytochrome P450-dependent enzymatic activities such as ethoxyresorufin *O*-deethylation (EROD). Therefore, measurement of EROD activity has been adopted as a valuable indicator allowing the detection of PAHs at presumably toxic levels (Van Veld et al., 1990; Addison et al., 1994).

PAHs have served as markers of oil pollution (Ogata and Miyake, 1979) and some PAH are known to be carcinogens (Andelman and Snodgrass, 1974). Petroleum hydrocarbons have been reported to cause structural damage to the gills in fish (Poirier et al., 1986; Correa and Garcia, 1990; Engelhardt et al., 1981; Prasad, 1991). PAHs tend to adsorb to organic or inorganic matter in sediments, where they are trapped in long-term reservoirs. Although only a portion of sediment-adsorbed PAHs are readily available to marine organisms, there is substantial uptake of these compounds by benthic fish through diet, through exposure to contaminated water in the benthic boundary layer, and through direct contact with sediment (Johnson et al., 2002). Benthic invertebrate preys are particularly important sources of PAH exposure for marine fishes, since PAHs are bioaccumulated in various marine invertebrates (Varanasi et al., 1989,

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1992; Meador et al., 1995).

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.

The aromatic hydrocarbons are the most water soluble components of crude oil, but their high volati- lity offsets their solubility when spills occur onto the surface of the sea (Gerlach, 1981; NeV, 1990). Despite this wealth of data there are few studies on the effects on the physiology of benthic organisms exposed to phenanthrene *in vivo*. Although Stickle et al. (1982) and Stephens et al. (2000) have already reported that high concentrations of oil in aqueous suspension disrupted osmoregulation in euryhaline fish, the present study is the first to investigate the effect of waterborne phenanthrene on a benthic fish (*Paralichthys olivaceus*).

The objectives of this research were to define gill response in CYP450 system in the olive flounder (*P. olivaceus*) exposed to different concentrations of phenanthrene.

Materials and Methods

Experimental animals

Olive flounder (mean±SD weight, 51±4.3 g) were obtained from a commercial dealer, transferred to a maintenance tank and kept at 20±1℃. Fish were acclimated to the laboratory condition for 2 weeks prior to experiment. Fish were maintained on a 12 h light/dark cycle.

Exposure conditions

Test chambers (glass aquarium, 120 L capacity) were filled with 80 L of sea water. Water characteristics, measured by the method described in APHA (1995), were as follows; pH 8.03±0.4, temperature 20±1℃, salinity 31.8±0.7 PSU and dissolved oxygen 7.5-7.8 mg/L. Phenanthrene was initially dissolved in ethanol (Sigma Chemical, St. Louis, MO) to obtain initial stock solution. Prior to introduction of fish to a test chamber, phenanthrene (>96% purity, Sigma Chemical, St. Louis, MO) working solution were mixed with filtered seawater to attain a nominal concentration of 0.5, 1.0 and 2.0 μM. The working solutions were stirred 8-16 hr prior to use. The phenanthrene dose was renewed every other day along with the seawater. Three test chambers were used for each treatment and each chamber contained 10 fish under semi-static conditions with airstones to maintain dissolved oxygen levels greater than 75% saturation.

Sampling

At the end of experiment, fish were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO) and gill microsome were isolated 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℃, supernatant harvested, and centrifuged at 100,500×g for 60 min using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Italy) at 4℃ to obtain a microsomal pellet. The pellet was resuspended in 0.1 M phosphate buffer (1mM Na_2EDTA, 1mM dithiothreitol (DTT), 20% glycerol, pH 7.4).

Cytochrome P450 and dealkylase assays

Cytochrome P450 content was determined by carbon monoxide-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). Gill 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); MgSO4, 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. The activity was determined at 25℃ using 25 μL postmitochondrial supernatant in the reaction mixture. The penthoxyresorufin *O*-deethylation (PROD) activity was assayed in the same systems as EROD. Enzyme activities were calculated using the fluorescence increase induced by the addition of resorufin. Protein concentration was determined by Bradford (1976), using a bovine serum albumin (Sigma, USA) as a standard.

Statistical analysis

Statistical analysis of the results was performed with the aid of SPSS/PC+ statistical package. ANOVAs

and the Duncan's test for multiple comparisons were used to test the significant differences between the control and treatment (Duncan, 1955).

Results and Discussion

We investigated the content of gill CYP450 in olive flounder exposed to phenanthrene for 2 or 4 weeks (Fig. 1). Following 2 weeks exposure, the phenanthrene exposed groups (1.0 μM and 2.0 μM) had significantly higher gill CYP450 contents compared to the control group ($P<0.05$). Compared with the values found in the control group, the values were about three times higher for gill cytochrome P450 contents in olive flounder exposed to 2.0 μM phenanthrene at 4 weeks ($P<0.001$). Fig. 2 shows the effect of phenanthrene on gill EROD activity in olive flounder. Following 4 weeks, gill EROD activity was increased significantly ($P<0.01$) by 2.5 and 2.3 fold at the 1.0 μM and 2.0 μM, respectively compared to initial control activity, while gill PROD activity was not induced at any treatment-groups (Fig. 3). The laboratory studies conducted by Haasch et al. (1989) and Skaare et al. (1991) have also established the expression of cytochrome P-4501A1 and EROD activity after the exposure of various pollutants. Although, monitoring of cytochrome P-4501A1 and its EROD activity have been widely used in aquatic environment as a biomarker, the effect of phenanthrene on these parameters has not yet been established.

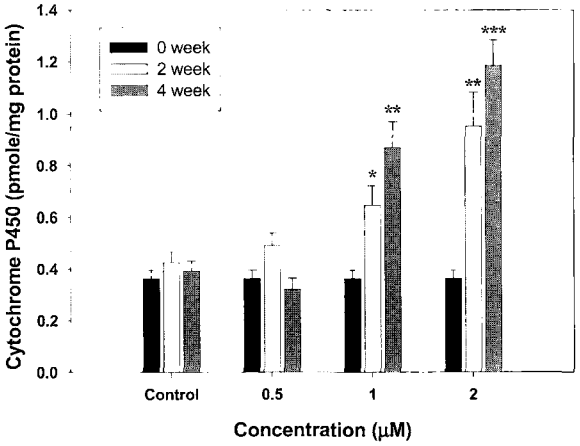

Fig. 1. Cytochrome P450 content (pmol/mg protein) of gill microsome in olive flounder (*Paralichthys olivaceus*) exposed to different levels of phenanthrene for 4 weeks. Values represent means and S.E. (n=10/treatment). Statistically significant differences between exposed fish and control fish: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

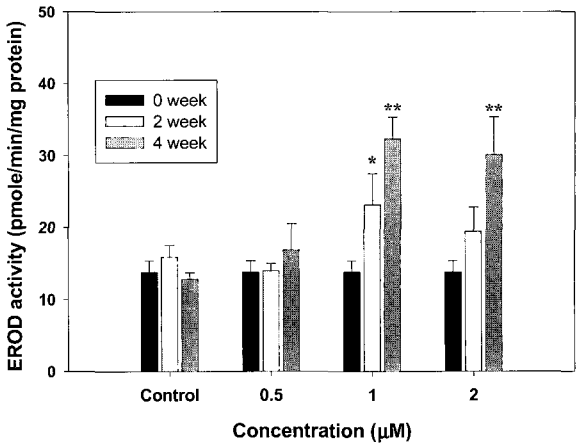

Fig. 2. EROD activity (pmole/min/mg protein) of gill microsome in olive flounder (*Paralichthys olivaceus*) exposed to different levels of phenanthrene for 4 weeks. Values represent means and S.E. (n=10/treatment). Statistically significant differences between exposed fish and control fish: $^{*}P<0.05$, $^{**}P<0.01$.

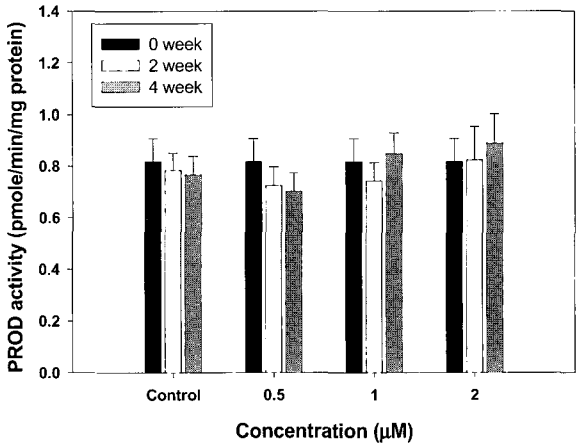

Fig. 3. PROD activity (pmole/min/mg protein) of gill microsome in olive flounder (*Paralichthys olivaceus*) exposed to different levels of phenanthrene for 4 weeks. Values represent means and S.E. (n=10/treatment).

Cytochrome P4501A1 has been localized in the gills of trout (*Oncorhynchus mykiss*) and scup (*Stenotomus chrysops*) where it is abundant in pillar cells and vascular endothelium but absent from the epithelium (Miller et al., 1989; Stegeman et al., 1991). Thus, toxicant exposure could potentially have profound effects on the gill's ability to metabolize regulatory molecules in the circulation or to alter the profile of regulatory products secreted by the gill.

Despite previous investigations carried out on this subject, some toxicological response in fish remain unknown, revealing in particular, the lack of data concerning the stress mechanism, as well as biotransformation and genotoxic responses. Biotransformation studies in fish reported that PAH exposure induces cytochrome P450-dependent enzymatic activities such as ethoxyresorufin *O*-deethylation (EROD).

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 they are non-responsive to PB-type compounds (Stegeman et al., 1990). Brown (1992) studied a large series of gas chromatograms of the PCB residues in 32 species of teleost fish. CYP2B-like alteration patterns were observed in only four species of his study. In the present study, insignificant PROD induction was observed in the phenanthrene-treated olive flounder, indicating that phenanthrene seems not to induce gill CYP2B class isozyme.

The results presented in this paper clearly indicates that gill cytochrome P450 and dealkylase activity in the olive flounder exposed for 4 weeks to phenanthrene are induced. 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. These results indicate that CYP450 and EROD activity are useful as a diagnostic for phenanthrene exposure in aquatic organisms.

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

Acknowledgements

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