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Biochemical Changes of Enzymatic Defense System after Phenanthrene Exposure in Olive Flounder, *Paralichthys olivaceus*

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Summary

The objective of this study was to investigate the early biological response in the olive flounder exposed to sub-lethal concentrations of waterborne phenanthrene (0.5, 1 or 2 μM). The fish were exposed for 4 weeks and we analyzed their enzymatic defense system, antioxidant and phase II enzyme activities, to evaluate the chronic exposure toxicity of phenanthrene. Waterborne phenanthrene affected antioxidant enzymes and glutathione-mediated detoxification as an enzyme defense system. Hepatic, gill and kidney glutathione reductase as well as glutathione S-transferase, and catalase activities were markedly elevated after two or four weeks of exposure. These enzyme activities of the olive flounder, *Paralichthys olivaceus*, seem to be a convenient approach for monitoring pollution in coastal areas against polycyclic aromatic hydrocarbon pollution including phenanthrene.

Key words

Antioxidant enzymes • Detoxification • Olive flounder • Phase Ⅱ enzyme • Phenanthrene

Introduction

In an attempt to define and measure the effects of pollutants on the aquatic ecosystem, biomarkers and bioindicators have attracted a great deal of interest. The principle of the biomarker approach is to analyze the organism's physiological or biochemical response to pollutant exposure.

Due to human influences, polycyclic aromatic hydrocarbons (PAHs) are becoming ubiquitous in estuarine and coastal areas and are commonly found in the marine sediment, water and tissues of marine organisms (Kennicutt *et al.* 1988, Wade *et al.* 1988, McElroy *et al.* 1989). In the aquatic environment, these hydrophobic organic chemical contaminants become rapidly associated with particles in the water and are deposited in sediments, thus consumed by benthic organisms (McElroy *et al.* 1989). According to Moon *et al.* (2001), sediments and organisms in Korean coastal areas are also contaminated with a wide range of PAHs. 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

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plants, aluminum plants, iron and steel works, foundries, municipal incinerators, synfuel plants, and oil shale plants (U.S. EPA 1987).

Phase Ⅱ reactions are biosynthetic reactions in which the foreign compound or a phase I-derived metabolite are covalently linked to an endogenous molecule (Sipes and Gandolfi 1986). Antioxidant enzymes thus play a crucial role in maintaining cell homeostasis. These enzymes have been proposed as biomarkers of contaminant-mediated oxidative stress in a variety of marine organisms, and their induction reflects a specific response to pollutants (Cossu *et al.* 1997). Oxidant-mediated toxicities may result if these enzymes are inhibited, enzymatic inactivation may occur if the system is impaired. Earlier usefulness of glutathione-dependent enzymes and other antioxidant enzymes as markers for oxidative stress in fish, has been reported by many workers (Rodriguez-Ariza *et al.* 1993, Hasspieler *et al.* 1994). Hence, no comprehensive phenanthrene-induced changes have hitherto been established. Recently, Jee *et al.* (2004) and Jee and Kang (2003) reported a toxic effect of waterborne phenanthrene on basic physiological impact and acetylcholinesterase activity. However, the information of phenanthrene on aquatic animals is not clear. In this study, we tried to elucidate the effect of waterborne phenanthrene by chronic exposure on phase II enzyme (GST) and antioxidant enzyme (GR, GPx, CAT) activities in gill, liver and kidney in the flounder (*Paralichthys olivaceus*), one of the most important aquacultured fish in Asian countries including Korea and Japan.

Methods

Healthy, cultured olive flounder (*P. olivaceus*), 51±4.3 g mean body weight, were selected from a local fish farm in Pohang, Kyongbook, Korea. Fish were acclimated to the laboratory for two weeks prior to the experiment. Fish were maintained on a 12 h light/dark cycle at all times. During this conditioning period, fish were fed with extruded pellet food (Jeilfeed Co., Korea; crude protein > 52 %, fat > 7 %, fiber < 4 %, calcium >1.2 %, phosphorus > 2.7 % and ash < 17 %, according to the manufacturer's specifications). The food was given to all groups of fish twice a day (2 % body weight for each meal at approximately 08:30 and 16:00 h).

Test chambers (glass aquaria, 120 l capacity) were filled with 80 l of sea water. Water characteristics, measured by the method described in APHA–AWWA–WPCF (1998), were as follows: pH 8.03±0.4; temperature 20±1 °C; salinity 31.8±0.7 ‰ 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 test aquaria, solutions of 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, 2.0 μM. The working solutions were stirred 8-16 h prior to usage. The phenanthrene dose was renewed every second day along with the seawater. Three test chambers were used for each treatment and each chamber contained ten fish. The conditions in the chambers were semi-static with airstones in order to maintain dissolved oxygen levels greater than 75 % saturation.

At the end of each period (at 2 and 4 weeks) fish were anesthetized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO) and weighed. Livers, gill and kidney were isolated from the experimental fish and homogenized in four-five volumes of ice cold buffer (50 mM TRIS, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, pH 7.5) with several passes of a teflon pestle (099C K4424, Glas-Col, USA). The homogenates were centrifuged (10 000 *g* for 20 min, MIKRO 22R, Hettich, Germany) at 4 °C, and the supernatant obtained was further centrifuged at (100 500 *g* for 60 min, T-1190, Kontron Instruments, Milano, Italy). The 105 000 *g* supernatant was used in analyses of cytosolic enzymes. All preparatory steps were carried out at 4 °C.

Cytosolic glutathione-S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al.* 1974). Briefly, the reaction contained 0.2 M potassium phosphate (pH 6.5), 10 mM glutathione, 10 mM 1-chloro-2,4-nitrobenzene with cytosolic protein. After 5 min reactions at room temperature, absorbance was determined on a spectrometer at 340 nm. The activity of glutathione peroxidase was assayed at 37 °C and 340 nm by calculating the difference in absorbance values during the oxidation of NADPH (Beutler 1984). Glutathione reductase activity was assayed at 37 °C and 340 nm by following the oxidation of NADPH with oxidized glutathione (GSSG). Tris-HCl (1 M), 5 mM EDTA (pH 8.0), and 10 mM H_2O_2 and H_2O were mixed and the rate of H_2O_2 consumption at 230 nm and 37 °C was measured for quantitative determination of catalase activity (Beutler 1984). An extinction coefficient for

H_2O_2 at 230 nm was used to calculate the activity of the enzyme.

Statistical analyses were performed using SPSS/PC+ statistical package. Significant differences between groups were determined using one-way ANOVA and Duncan's test for multiple comparisons or the Student's t-test for two groups (Duncan 1955). Significance level was set at $P<0.05$ or $P<0.01$.

Table 1. Alteration in liver, gill and kidney cytosolic glutathione-S-transferase activity (μmol/min/mg protein) of the olive flounder, *Paralichthys olivaceus* exposed to phenanthrene for 4 weeks

Organs	Exposure period	Phenanthrene concentration			
		Control	0.5 μM	1 μM	2 μM
Liver	2 weeks	0.65±0.07 [a]	0.67±0.04 [ab]	0.93±0.07 [bc]	0.74±0.14 [abc]
	4 weeks	0.64±0.03 [a]	0.80±0.03 [ab]	0.83±0.10 [abc]	1.00±0.11 [c]
Gill	2 weeks	0.29±0.02 [a]	0.39±0.03 [ab]	0.55±0.08 [b]	0.88±0.08 [c]
	4 weeks	0.36±0.05 [ab]	0.46±0.05 [ab]	0.52±0.08 [b]	0.93±0.07 [c]
Kidney	2 weeks	0.23±0.02 [a]	0.33±0.04 [a]	0.55±0.08 [bc]	0.58±0.07 [c]
	4 weeks	0.25±0.03 [a]	0.38±0.05 [ab]	0.52±0.08 [bc]	0.56±0.09 [bc]

Data are represented as mean ± S.E.M. (n=10). Means within each column followed by the same letter are not significantly different ($P>0.05$).

Table 2. Alteration in liver, gill and kidney cytosolic glutathione reductase activity (μmol/min/mg protein) of the olive flounder, *Paralichthys olivaceus* exposed to phenanthrene for 4 weeks

Organs	Exposure period	Phenanthrene concentration			
		Control	0.5 μM	1 μM	2 μM
Liver	2 weeks	1.46±0.19 [a]	1.50±0.05 [a]	2.65±0.35 [b]	3.01±0.30 [b]
	4 weeks	1.40±0.10 [a]	1.69±0.09 [a]	2.74±0.56 [b]	2.85±0.39 [b]
Gill	2 weeks	4.42±0.33 [ab]	5.30±0.47 [ab]	8.62±0.81 [d]	7.77±0.54 [cd]
	4 weeks	4.07±0.33 [a]	6.14±0.54 [bc]	8.52±0.78 [d]	7.41±0.83 [cd]
Kidney	2 weeks	2.92±0.39 [abc]	2.55±0.35 [ab]	3.62±0.15 [bcd]	3.39±0.44 [abcd]
	4 weeks	2.72±0.29 [abc]	2.37±0.23 [a]	3.75±0.46 [bc]	4.43±0.52 [d]

Data are represented as mean ± S.E.M. (n=10). Means within each column followed by the same letter are not significantly different ($P>0.05$).

Results

Activities of GST in the liver, gill and kidney of the olive flounder exposed to phenanthrene are shown in Table 1. The time course of the induction of olive flounder hepatic, gill and kidney cytosolic GST activity toward CDNB as substrate by waterborne phenanthrene (>1.0 μM) indicates a marked elevation at 2 and 4 weeks. Among the tissue GST enzyme, gill GST activity in olive flounder (2 μM) increased significantly after 4 weeks ($P<0.01$).

At 2 and 4 weeks, hepatic and gill cytosol GR activities in flounders exposed to phenanthrene (>1.0 μM) were significantly increased compared to the control (liver, $P<0.05$; gill, $P<0.01$), while kidney cytosol GR activity in flounders exposed to phenanthrene was not induced in any treatment-groups at 2 weeks (Table 2). At 4 weeks, kidney cytosol GR activity in flounders exposed to phenanthrene (2.0 μM) increased significantly ($P<0.01$) by 60 % compared to control activity.

The activities of GPx in the liver and gill and kidney of flounders exposed to phenanthrene are given in Table 3. The time course of the induction of olive flounder hepatic, gill and kidney cytosolic GPx activity by waterborne phenanthrene (>1.0 μM) showed a marked elevation at 2 and 4 weeks. In case of hepatic GPx, 2.0 μM of phenanthrene induced a remarkable increase ($P<0.01$).

In this study, we investigated the hepatic, gill and kidney catalase activity in the flounder exposed to phenanthrene for 2 or 4 weeks (Table 4). After 2 weeks of exposure, significantly higher hepatic cytosol catalase activity compared to the control group ($P<0.05$) was observed in the phenanthrene-exposed groups (2.0 μM). Compared with the values found in the control group, the values were about two times higher for hepatic cytosol catalase activity in flounders exposed to 2.0 μM phenanthrene at 4 weeks ($P<0.01$). However, there was no significant variation in gill cytosol activity between control and treatment group except in the 2.0 μM phenanthrene-exposed group for 4 weeks ($P<0.05$). A significantly increased rate of kidney cytosolic catalase activities was reported in fish exposed to phenanthrene (>1.0 μM) with respect to the control group. The values are significant at $P<0.01$.

Table 3. Alteration in liver, gill and kidney cytosolic glutathione peroxidase activity (nmol/min/mg protein) of the olive flounder, *Paralichthys olivaceus* exposed to phenanthrene for 4 weeks

Organs	Exposure period	Phenanthrene concentration			
		Control	0.5 μM	1 μM	2 μM
Liver	2 weeks	17.34±2.10 ^{a}	23.51±4.48 ^{abc}	41.39±10.77 ^{cd}	39.47±6.38 ^{cd}
	4 weeks	16.39±1.90 ^{a}	20.73±4.60 ^{ab}	36.27±6.25 ^{bcd}	47.53±6.36 ^{d}
Gill	2 weeks	9.76±1.35 ^{a}	13.54±1.79 ^{a}	20.73±4.60 ^{ab}	26.90±3.06 ^{c}
	4 weeks	8.63±1.15 ^{a}	12.53±0.99 ^{a}	27.95±3.44 ^{c}	25.60±3.13 ^{c}
Kidney	2 weeks	12.53±0.99 ^{a}	13.54±1.79 ^{ab}	20.73±4.60 ^{bc}	26.90±3.06 ^{c}
	4 weeks	13.54±1.79 ^{ab}	16.65±1.09 ^{ab}	25.24±2.68 ^{c}	25.22±2.42 ^{c}

Data are represented as mean ± S.E.M. (n=10). Means within each column followed by the same letter are not significantly different ($P>0.05$).

Table 4. Alteration in liver, gill and kidney cytosolic catalase activity (mmol/min/mg protein) of the olive flounder, *Paralichthys olivaceus* exposed to phenanthrene for 4 weeks

Organs	Exposure period	Phenanthrene concentration			
		Control	0.5 μM	1 μM	2 μM
Liver	2 weeks	32.92±2.56 ^{ab}	43.06±5.76 ^{abc}	44.17±7.30 ^{bc}	72.00±5.17 ^{d}
	4 weeks	24.92±4.60 ^{a}	34.29±5.46 ^{ab}	57.76±8.07 ^{cd}	66.60±6.95 ^{d}
Gill	2 weeks	37.24±5.81 ^{ab}	31.25±3.17 ^{a}	44.17±7.30 ^{bbc}	51.30±7.23 ^{bc}
	4 weeks	32.30±4.21 ^{a}	34.29±5.46 ^{ab}	46.49±5.66 ^{abc}	55.41±4.70 ^{c}
Kidney	2 weeks	13.89±1.32 ^{a}	20.34±2.44 ^{ab}	25.60±3.13 ^{abc}	34.29±5.46 ^{bcd}
	4 weeks	12.80±1.83 ^{a}	26.90±3.06 ^{abc}	46.49±5.66 ^{d}	42.43±4.84 ^{cd}

Data are represented as mean ± S.E.M. (n=10). Means within each column followed by the same letter are not significantly different ($P>0.05$).

Discussion

Biotransformation systems are generally regarded as consisting of two subsystems: phase I and phase II systems. In phase I metabolism, most lipophilic chemicals are oxidized by the cytochrome P450-dependent monoxygenase system by introduction of hydroxyl groups into the chemical, yielding a suitable substrate for the phase II reaction. In the phase II system, the metabolite may be conjugated with various polar endogenous substrates such as glutathione, glucuronic acid, and sulfate by the transferase to form more soluble products that are readily excreted *via* the bile or kidney (Benson and Di Giulio 1992). The main enzymes which detoxify reactive oxygen species in all organisms are glutathione peroxidase (EC 1.11.1.9), peroxidase (EC 1.11.1.7), and catalase (EC 1.11.1.6), all of them being abundant in fish tissue (Di Giulio *et al.* 1993). These enzymes can be induced by reactive oxygen species and they may be useful indicators of oxidative stress. The phase II system and endogenous cellular glutathione (GSH) as environmental indicators have received relatively little attention (Stein *et al.* 1992). Induction of antioxidants can provide sensitive early warning signals of incipient oxidative stress (Benson and Di Giulio 1992). Therefore, in this study, GST and some antioxidative enzyme activities were investigated in the liver, gill and kidney of the olive flounder, *P. olivaceus*, exposed to waterborne phenanthrene.

GST has been found in all organisms in which it had been investigated (Stenersen *et al.* 1987, Dierickx 1984), and it seems likely that it is ubiquitous. The activity of GST is known to increase in rats exposed to PCBs (Kamohara *et al.* 1984). The present findings corroborated the observation of Davies (1985) and Fair (1986) for fish exposed to chlorothalonil and benzo(a)pyrene, respectively. Similarly, cadmium-exposure of rainbow trout for 4 weeks caused an initial decrease in hepatic and nephrotic GST in a previous study, followed by a net increase in hepatic GST activity (Förlin *et al.* 1986). In this study, the induction of olive flounder hepatic, gill and kidney cytosolic GST activity toward CDNB as substrate by waterborne phenanthrene (>1.0 μM) indicates a marked elevation at 2 and 4 weeks. These results demonstrated that GST enzyme activities were significantly altered in the flounder by treatment to waterborne phenanthrene. Oxidative lesions have recently been related to liver tumor formation in fish from polluted environments (Malins and Haimanot 1991). However, Gallagher *et al.* (1996) reported that GST detoxifies a number of environmental carcinogens and epoxide intermediates. Thus, the GST assay was suggested as a useful tool for biomonitoring oxidative stress (Di Giulio *et al.* 1993). The alteration in GST activity due to phenanthrene suggests a stressed situation of the olive flounder in the present investigation.

The status of other antioxidant systems was variably affected by PAHs exposure (Niyogi *et al.* 2001, Veignie *et al.* 2004). In the current study, the activity of gill GR was significantly higher in the fish exposed to phenanthrene than in the controls. Similar results have been reported by Stephensen *et al.* (2003), who observed higher hepatic GR activity in fish exposed to PAHs under laboratory conditions as well as higher activities of GR in fish inhabiting a PAH-polluted harbor.

Glutathione peroxidase (GPx) is the most important peroxidase for the detoxification of hydroperoxides. It catalyzes the glutathione-dependent reduction of hydroperoxides and of hydrogen peroxide. Glutathione peroxidase activity may be induced by environmental pollutants. The activity increases together with glutathione reductase in the rainbow trout after injection of tetrachlorobiphenyl (Otto and Moon 1995) and in the carp after exposure to copper, but not to paraquat (Matkovics *et al.* 1987). In this study, the activities of GPx in the liver, gill and kidneys of flounders showed the time course induction of waterborne phenanthrene (>1.0 μM), indicated marked elevation at 2 or 4 weeks. GPx enzyme has been postulated to protect erythrocytes from damage by H_2O_2 and responsible for reduction of lipid hydroperoxides. Therefore, it is hypothesized that this enzyme may protect tissues against oxidative damage due to lipid peroxidation. The liver is a major site of detoxification and the first target of ingested oxidants, and is considered to be a very important tissue in the study of GPx protective role from lipid peroxidation.

Catalase primarily occurs in peroxisomes. Its activity increases together with other peroxisomal enzymes in the fish liver upon exposure of animals exposed to bleached kraft mill effluents, suggesting that a general peroxisomal proliferation is induced (Mather-Mihaich and Di Giulio 1991). Some pollutants may inhibit catalase activity. High concentrations of copper inhibited catalase activity in the liver, gill and muscle, and 100 ppm $ZnSO_4$ in the gill and muscle (Radi and Matkovics 1988). In this study, when compared with the values found in the control group, the values were about

two times higher for hepatic cytosol catalase activity in flounders exposed to 2.0 μM phenanthrene at 4 weeks ($P<0.01$). However, there was no significant variation in gill cytosol activity between control and treatment group except 2.0 μM phenanthrene at 4 weeks ($P<0.05$). Kidney cytosolic catalase activities in fish exposed to phenanthrene (>1.0 μM) were significantly increased compared to those of the control group at 4 weeks ($P<0.01$).

We can conclude that the enzyme defense system is affected by phenanthrene because it causes significant changes in the activities of several antioxidant enzymes and glutathione-mediated detoxification enzyme in the liver, gill and kidney tissues of *Paralichthys olivaceus.*

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