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The Dose–Response Relationships for EROD and GST Induced by Polyaromatic Hydrocarbons in *Carassius auratus*

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Abstract Freshwater fish *Carassius auratus* were chosen as experimental animals, the hepatic biochemical responses to medium-term exposure of five PAHs were measured as ethoxyresorufin *O*-deethylase (EROD) activity (phase I) and glutathione *S*-transferase (GST) activity (phase II) to assess sub-lethal effects. The fold increases of EROD and GST activity were calculated and both increased in the order Fluoranthene < Fluorene < Benzo(b)fluoranthene < Benzo (g,h,i)perylene < Indeno(1,2,3-cd)-pyrene. The clear dose–response relationships were found for liver EROD and GST activity induced by PAHs. The enzyme EROD and GST in *Carassius auratus* were confirmed as useful biomarkers of exposure to both PAH and PAH-like compounds.

Keywords PAHs · Fish · Biotransformation · Dose–response relationships

In the aquatic environment, the exposure of living organisms to xenobiotics leads to interactions between these chemicals and biological systems, which may give rise to biochemical disturbances or/and adaptive responses (Masfaraud et al. 1992). Metabolism or biotransformation through the phase I (P450 monooxygenase enzymes) and phase II (conjugating enzymes) pathway are requisites for detoxification and excretion of lipophilic chemicals in aquatic animals (Goksøyr and Förlin 1992).

The cytochrome P450-dependent monooxygenase enzymes (CYPs) comprise a family of structurally and functionally related heme proteins which are involved in the oxidative metabolism of a broad range of substrates, including drugs and environmental chemicals (Nelson et al. 1996; Mansuy 1998). The cytochrome P4501A(CYP1A) is of central importance in the metabolism of many xenobiotics. Induction of hepatic mixed-function oxidase enzymes of phase I, especially CYP1A and associated ethoxyresorufin *O*-deethylase (EROD) activity, is considered a common indicator of exposure of fish to environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs) (Stephensen et al. 2003) and polychlorinated biphenyls (PCBs) (Hugla and Thome 1999).

In addition to enzymes that participate in the phase I of elimination of xenobiotics, most fish possess a second group of biotransformation enzymes referred to as conjugation or phase II enzymes, which are also induced by exposure to different type of pollutants (Stanic et al. 2006). Glutathione *S*-transferase (GST) are a family of enzymes important in the biotransformation of xenobiotics, being of crucial importance in the detoxification of a large number of substances, such as PAHs, PCBs, polychlorinated dibenzodioxins (PCDDs) and phenobarbital (George 1994).

Petroleum hydrocarbons contamination in China poses a particularly high risk because of its impact on the great diversity of aquatic life. In the muscles of the fish collected from fishpond of Pearl river delta, the total levels of PAHs ranged from 1.91 to 224.03 ng/g (wet weight) with a mean value of 49.59 ng/g wet weight (Kong et al. 2005), and the total PAHs ranged between 184 and 194 ng/g (dry weight) in the muscles of the fish from Mai Po Marshes Nature Reserve of Hong Kong (Liang et al. 2007). *Carassius*

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auratus is a major kind of economic fish and distribute abroad in various freshwaters in China. The aims of the present work, using *C. auratus*, were to assess the waterborne PAHs effects on biotransformation, measured as EROD activity (phase I) and GST activity (phase II) and to study their dose–response relationships.

Materials and Methods

Fluorene, fluoranthene, benzo(b)fluoranthene, benzo(g,h,i) perylene, indeno(1,2,3-cd)pyrene, nicotinamide adenine dinucleotide phosphate (NADPH), 3,3′-methylenebis-(4-hydroxycoumarin), CDNB (1-chloro-2,4-dinitrobenzene), resorufin (RF), ethoxyresorufin (ERF) and GSH (glutathione) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and the stated purities were >99.9%. Tris (hydroxymethyl aminomethane) and KCl were purchased from Nanjing Sunshine Biotechnology Co., LTD. (Nanjing, China) and their purities were >99%. Bovine serum albumin was purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China) and the purity was >98%. Coomassie brilliant blue G-250 (Ultra Pure Grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

Crucian (*C. auratus*) were obtained from Nanjing Institute of Fishery Sciences. The average length and weight were 15.28 cm (±0.39 cm) and 40.55 g (±1.25 g) respectively. The fish were acclimated for 2 weeks in dechlorinated municipal water.

Chemical treatments were delivered via intraperitoneal injection at dosages of 0.1, 1.0, 2.0, 5.0 and 10.0 (or 8.0) mg/kg PAHs dissolved in corn oil. Control animals received corn oil only. Dose range was based on range-finding experiments (no lethal effect at the highest exposure dosage) and the lowest dosage was based on the observed fish burdens in the field in China (Kong et al. 2005; Liang et al. 2007). Fish were weighed before injection to determine the volume of dosage per kilogram body mass of each fish. Masses and dosages were recorded. Fish were kept three to five to a 30-L glass tank in dechlorinated municipal water under constant aeration and were not fed throughout the experiment. A 50% water change was performed every other day. Water temperatures ranged from 20 to 22°C.

After a 15-day exposure, fish were killed by cervical transection and livers were collected. Livers were carefully dissected, washed in 0.15 mol/L of KCl, weighed, and stored at −80°C until preparation of microsomes. Liver samples were homogenized in 5 volumes of buffer (0.25 mol/L sucrose, 0.1 mol/L Tris–HCl, 1 mmol/L EDTA, pH = 7.4) and centrifuged at 9,000 r/min for 15 min. Supernatant was collected and stored at −80°C. EROD activity was quantified using 96-well plates described by Chen et al. (1999). The reaction mixture consisted of 140 μl buffer (0.1 mol/L Tris, 0.15 mol/L KCl, pH 8.0), 10 μl of 2 μmol/L 7-ethoxyresorufin and 10 μl microsomes. The reaction was initiated at 25°C by the addition of 40 μl of 2.1 mg/ml NADPH. O.D. values were determined at 572 nm. Results are reported as nmol resorufin/min/mg protein.

GST activity was measured following the general methodology described by Habig and Jakoby (1981) adapted to microplate reader (Frasco and Guilhermino 2002). The reaction mixture consisted of 100 μl of 0.1 mmol/L potassium phosphate, 10 μl of 1.0 mmol/L CDNB, 10 μl of 1.0 mmol/L GSH and 880 μl H_2O. One hundred and sixty microliters of reaction mixture and 40 μl of cytosol were incubated at 30°C for 30 s. H_2O instead of cytosol was used in blank wells. The increase in absorbance was recorded at 340 nm wavelength for 2 min in 12 s intervals. Vmax values were calculated using the extinction coefficient of 9,600 mol/L/cm to calculate the rate of GST activity. Final numbers are reported as nmol GSH/mg protein/min. Each sample was measured in triplicate.

Protein concentrations were determined with the Coomassie Protein Assay Kit (Bradford 1976), with bovine serum albumin as standard. Measurements were done on a microplate reader at 595 nm.

For each biomarker, data were expressed as mean ± SD. Data from different treatments were compared by one-way analysis of variance (ANOVA) and statistical different treatments were identified by Dunnett's *t* test. All statistical analyses were performed using the SPSS statistical package (ver. 12.0, SPSS Company, Chicago, IL, USA).

Results and Discussion

We observed fish toxic responses during the course of the test. No significant differences in the behavior of the fish between the lower dosage exposures and the corresponding controls were observed for all the five chemicals. Nevertheless, under the higher exposure dosages of PAHs, the fish appeared to be excited and jump severely within 3 h and the gills of the fish looked a little redder in comparison with control after 24 h of exposure. The phenomena are more obvious for the fish injected with benzo(b)fluoranthene and indeno(1,2,3-cd)pyrene. The fish appeared to be less active gradually and crouch in the corner of aquaria accompanying the exposure length extension. At last, the fish showed no response at the change of surrounding. In addition, when the injection dosages were more than 1 mg/kg, the obvious histopathological lesions were observed. The colors of fish viscera changed to fuscous, the

livers enlarged, and the borders of liver, kidney and cholecyst were blurred.

The CYP1A induction measured either by immunodetection or through its catalytic activity is probably the best-studied biomarker. Hence, EROD activity has been widely used as a biomarker for fish exposure to substances that bind to the aryl hydrocarbon (Ah) receptor (Teles et al. 2005). Both naphthalene (NAP) and β-naphthoflavone (BNF) revealed to be strong biotransformation (phase I) inducers. After 3-day exposure, liver EROD activity was significantly increased by all NAP and BNF tested concentrations. At 6 and 9 days, liver EROD activity was significantly induced mainly by the highest NAP and BNF concentrations (Pacheco and Santos 2002).

The in vivo effects of PAHs on hepatic EROD and the total protein data for the livers are presented in Fig. 1. Low dosages of fluorene and fluoranthene (0.1, 1.0 and 2.0 mg/kg) did not induce obvious effects on EROD. However, EROD activity was significantly induced by exposure to higher dosages of the two chemicals (Fig. 1a, b). No significant difference was observed at lower dosages of benzo(b)fluoranthenel (0.1 mg/kg) (Fig. 1c), while significant differences were found at all the doses tested for both benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene (Fig. 1d, e). However, indeno(1,2,3-cd)pyrene at the highest dosage (10.0 mg/kg) resulted in a decrease of fold induction.

Liver GST are involved in the biotransformation of several pollutants, therefore an induction of GST activity has been widely used as environmental biomarker. In this study, GST activity was significantly altered by exposure to high dosages of fluorine (2.0, 5.0 and 10.0 mg/kg) (Fig. 2a). No significant difference was observed at lower dosages of fluoranthene, benzo(g,h,i)perylene and benzo(b)fluoranthenel (0.1 mg/kg), while significant differences were found for higher dosages tested (Fig. 2b, c, d). It was similar to EROD response pattern, GST activity was significantly induced by all indeno(1,2,3-cd)pyrene tested dosages but 0.1 mg/kg and the fold induction declined at the highest exposure dosage (10.0 mg/kg), i.e., both EROD and GST induced by indeno(1,2,3-cd)pyrene exhibited bell shaped

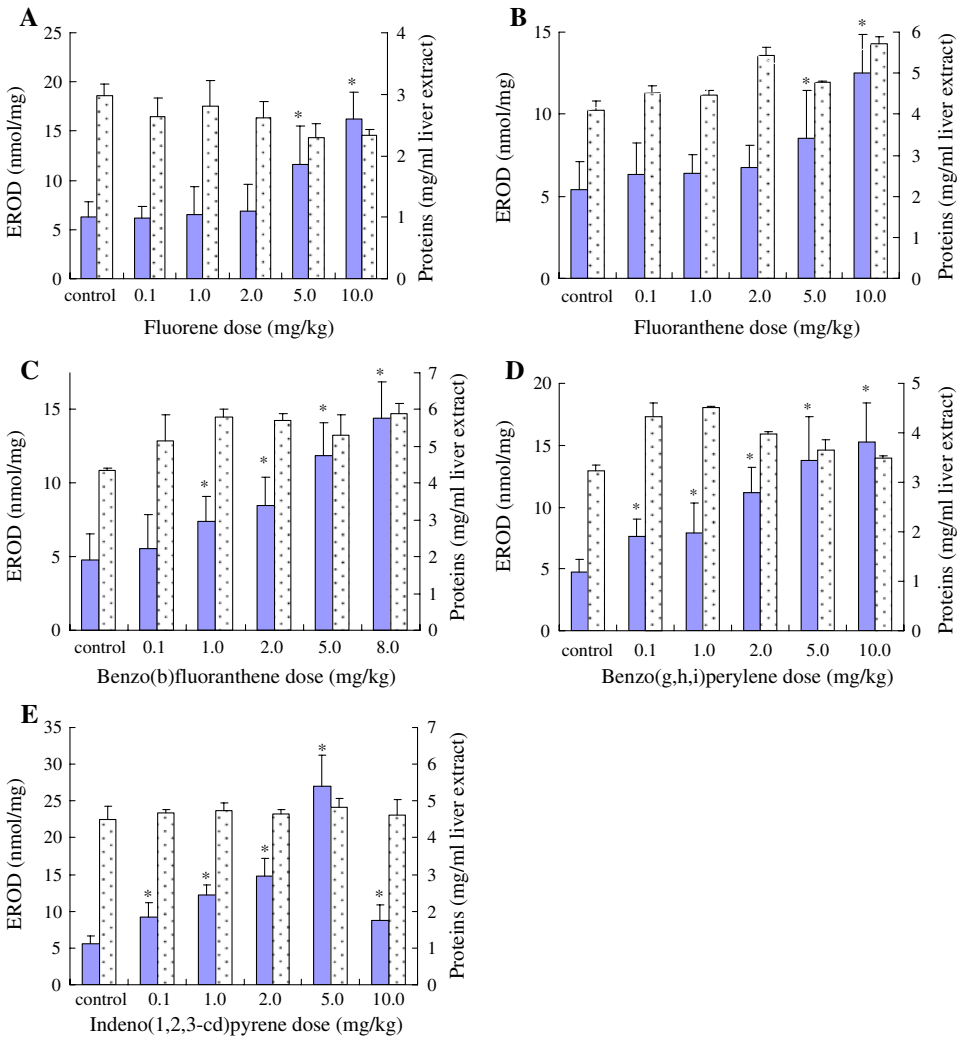

Fig. 1 Liver EROD activities (▮) and protein contents (▒). *Bars* indicate standard error of the mean. *Asterisks* indicate values that are significantly higher than control values ($p < 0.05$)

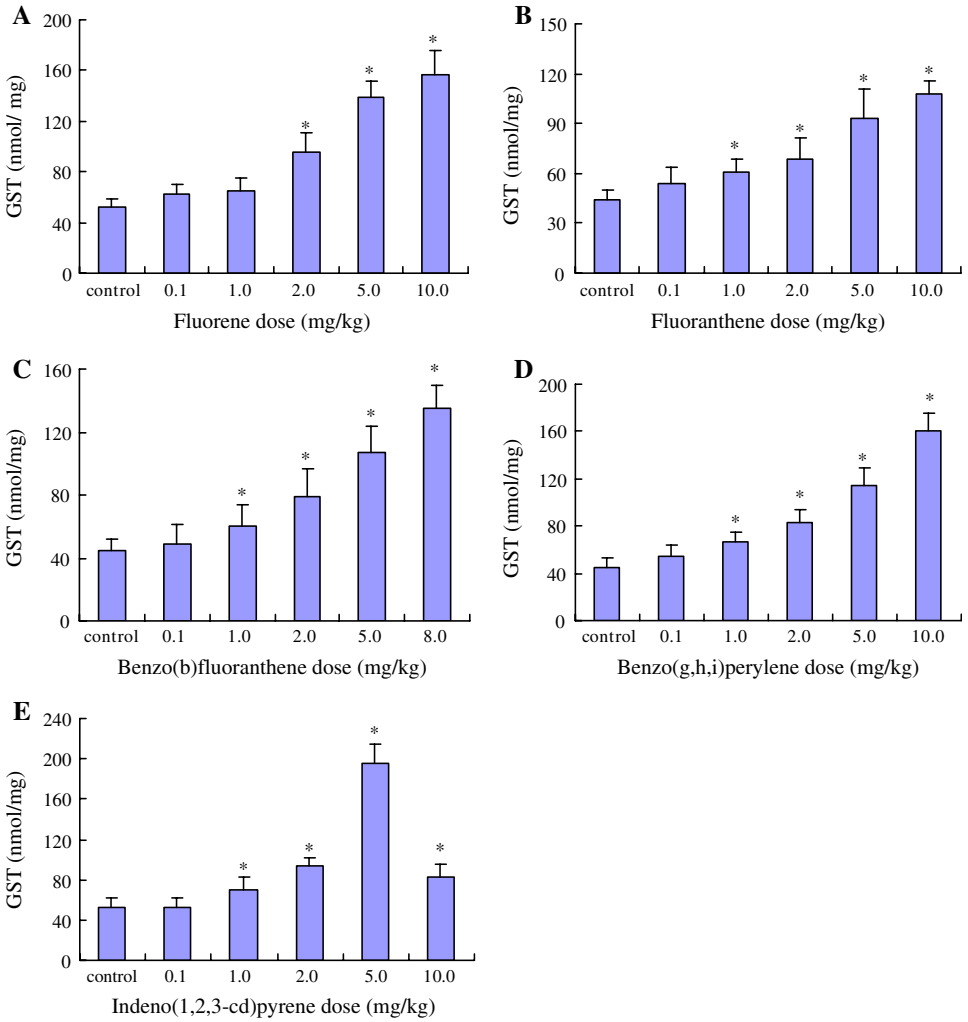

Fig. 2 Liver GST activity. *Bars* indicate standard error of the mean. *Asterisks* indicate values that are significantly higher than control values ($p < 0.05$)

dose–response curves. Bell-shaped curves have been reported for various in vitro and in vivo systems after exposure to PAHs (e.g., Kennedy et al. 1996; Delesclue et al. 1997; Bosveld et al. 2002). Although the mechanism resulting in decreased EROD or GST activity has not been completely defined, it is likely that high concentrations of the inducer inhibit or inactivate the induced enzyme (Voorman and Aust 1987).

The quantitative relationships between the dosages of contaminants and the fold increases of enzymatic activity were investigated (see Table 1). In Table 1, x is the exposure dosage, y is the enzymatic fold increase, n is the number of observations, r^2 is the square of the correlation coefficient, s is the standard error, F is the mean square ratio and p is the probability greater than the F value. From Table 1, a significant positive correlation can be found for every chemical within a designated dosage range, whether EROD or GST activity is used as response endpoints ($p < 0.05$).

In order to compare the biochemical disturbances of different PAHs, the fold increases of EROD and GST activity were calculated and listed in Table 2. Relative EROD and GST inducing potencies were based upon comparison of the observed highest fold increases of enzymatic activity. Both EROD and GST induction increased in the following order: Fluoranthene < Fluorene < Benzo(b) fluoranthene < Benzo(g,h,i)perylene < Indeno(1,2,3-cd) pyrene. The potency comparison based on the fold increases of EROD and GST activity at the same exposure dosage (5 mg/kg) showed consistent results (see Table 2).

The results in this study demonstrate that the five PAHs (having 3–5 fused aromatic rings) resulted in a significant change in hepatic EROD and GST activity in *C. auratus*. These results are supported by previous studies on other species, treated by different intraperitoneal PAHs injections. Benzo(a)pyrene displayed strong liver EROD and GST induction potency in *S. aurata* (Banni et al. 2008). Phenanthrene was found to induce a concentration-dependant formation of the enzyme EROD in the tilapia, *Oreochromis mossambicus*. The MFO system concerned with biotransformation of PAHs was highly active in *O. mossambicus* and tilapia were found to be eminently

Table 1 Dose–response relationships for EROD and GST activity

Chemicals	Dose range (mg/kg)	Dose–response relationships	n	r^2	s	F	P
Fluorene	0.1–10	EROD: $y = 0.173x - 0.111$	5	0.981	0.111	156	0.001
		GST: $y = 0.190x + 0.270$	5	0.889	0.311	24.1	0.016
Fluoranthene	0.1–10	EROD: $y = 0.120x + 0.069$	5	0.980	0.081	144	0.001
		GST: $y = 0.126x + 0.290$	5	0.949	0.135	55.8	0.005
Benzo(b)fluoranthene	0.1–8	EROD: $y = 0.227x + 0.255$	5	0.984	0.107	190	0.001
		GST: $y = 1.505x - 0.209$	5	0.991	0.088	313	0.000
Benzo(g,h,i)perylene	0.1–10	EROD: $y = 0.166x + 0.736$	5	0.854	0.319	17.6	0.025
		GST: $y = 0.240x + 0.307$	5	0.993	0.095	413	0.000
Indeno(1,2,3-cd)pyrene	0.1–5	EROD: $y = 0.660x + 0.535$	4	0.993	0.142	296	0.003
		GST: $y = 0.564x - 0.191$	4	0.989	0.155	180	0.005

Table 2 Data of fold increases of EROD and GST activity

Compounds	Highest fold induction			Fold induction at dosage of 5 mg/kg	
	EROD	GST	Dosage	EROD	GST
Fluorene	1.58	1.98	10	0.86	1.62
Fluoranthene	1.32	1.46	10	0.59	1.11
Benzo(b)fluoranthene	2.00	2.01	8	1.47	1.38
Benzo(g,h,i)perylene	2.21	2.63	10	1.89	1.58
Indeno(1,2,3-cd)pyrene	3.90	2.70	5	3.90	2.70

suited for PAH pollution biomonitoring in tropical coastal waters (Shailaja and Classy 2003). The similar results were obtained in vitro bioassay. The EROD inducing potency of 10 polycyclic aromatic hydrocarbons (PAHs) was measured in the H4IIE cells. Fluoranthene and benzo(g,h,i) perylene showed weak responses at the highest doses. The other PAHs, including indeno(1,2,3-cd)pyrene, benz(a) anthracene, benzo(a)pyrene, chrysene and benzo(k)fluoranthene showed full bell shaped dose–response curves (Bosveld et al. 2002). Liver microsomes from the fish *Dicentrarchus labrax*, treated with β-naphthoflavone (50 mg/kg) or benzo(a)pyrene (20 mg/kg), showed a 4–15-fold increase of EROD activity (Viarengo et al. 1997).

Enzymatic biotransformation is an important process responsible for the detoxication and elimination of xenobiotic in both vertebrates and invertebrates. The clear relationships between liver EROD and GST activity and exposure dosages of PAHs were found in this study. The enzyme EROD and GST in *C. auratus* were confirmed as useful biomarkers of exposure to both PAH and PAH-like compounds. However, this study investigated only fish biochemical responses to exposure of individual PAHs. In actual water bodies, aquatic organisms are not exposed to single substances but rather simultaneously to multiple mixtures of chemicals. Further research should be carried out in the field to qualify *C. auratus* as a suitable biomonitoring species in fresh waters.

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