Interaction on biomarkers in goldfish after co-exposure to 17β-estradiol and benzo(a)pyrene

YAN Zhenhua [1,a], LU Guanghua [1,b]

[1]Key Laboratory of Integrated Regulation and Resource Development on Shallow Lakes of Ministry of Education, College of Environment, Hohai University, Nanjing 210098, China

[a]email: hwahuer@hhu.edu.cn, [b]email: ghlu@hhu.edu.cn

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Abstract. The aquatic environment is challenged with complex mixtures of pollutants, which may produce synergistic or antagonistic effects in organisms, interacting on the established biomarkers. This study focuses on the interaction on biomarker responses in male goldfish (*Carassius auratus*) after co-exposure to 17β-estradiol (E2) and benzo(a)pyrene (BaP). Vitellogenin (Vtg), endogenous E2 and ethoxyresorufin-O-deethylase (EROD) were examined to assess the estrogenic effect, steroidogenesis, and metabolism capacity. Exposure to E2 or BaP alone significantly induced the production of Vtg and EROD, respectively. However, these inductions were markedly depressed by the co-exposed chemical, indicative of a reciprocal inhibiting interaction on Vtg and EROD. In addition, the E2-induced steroidogenesis were also suppressed by the coexisting BaP, while the steroidogenesis were not affected by BaP alone. Therefore, our results support a reciprocal inhibiting interaction on the established biomarkers on the estrogenic effect and metabolism capacity, and a one-way inhibition on the steroidogenesis pathway in goldfish after co-exposure to E2 and BaP.

Introduction

Biomarkers can provide very valuable information about types of exposure as well as exposure pressures in the field programs. For instance, induction of biotransformation enzymes in fish, such as cytochrome P4501A (CYP1A) and the related enzyme activity in a reaction called ethoxyresorufin-O-deethylase (EROD) activity are the well-documented biomarkers for assess exposures to the aryl hydrocarbon receptor (AhR) agonists, including polycyclic aromatic hydrocarbons (PAHs) in waters [1]. In addition, induction of the egg-yolk precursor vitellogenin (Vtg) in male or juvenile fish, via activation of the estrogen receptor (ER), is another biomarker that is frequently used to assess exposure to estrogenic chemicals and provides an early warning signal for exposure to ER agonists in the aquatic environment [2]. Hence, both of these established biomarkers have been extensively used for risk-assessments as well as for estimations of environmental exposures in environmental monitoring programs.

However, it is also noteworthy that aquatic environments serve as the ultimate sink for many environmental pollutants and aquatic species are continuously and increasingly subjected to complex cocktails of chemicals rather than individual chemicals in their natural habitats [1,3]. It means that the presence of other different classes of chemicals may affect the established biomarkers and further obscure the picture, leading to either underestimation or overestimation of the actual exposure situation. Therefore, it is important to address the interaction on biomarkers for more accurate and safer interpretations of monitoring data in situations of mixed exposure [4]. However, the knowledge of the interaction on these established biomarkers are still limited.

Hence, the purpose of the present study was undertaken to investigate the interaction on the established biomarkers in fish, Vtg and EROD, after co-exposure of male goldfish (*Carassius auratus*) to aromatic hydrocarbons and estrogens, reevaluating the combined effects of multiple

chemicals. Since 17β-estradiol (E2) and benzo(a)pyrene (BaP) were widely coexistent chemicals in aquatic environments, we selected them as the ER and AhR agonist, respectively. The blood and liver Vtg levels, serum E2 levels and EROD activity in liver were measured.

Material and methods

Chemicals. E2 (≥98% purity) and BaP (≥98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Their stock solutions were prepared in dimethylsulfoxide and stored at -20 °C. Test water was prepared using Millipore Milli-Q integral water purification system (Milford, USA).

Animals and exposure. Adult male goldfish (33.4±1.3 g, 17.0±3.2 cm) were obtained from the Nanjing Institute of Fishery Science (Nanjing, China) and acclimatized in dechlorinated municipal water for two weeks prior to the exposure. Fish were fed with pellet food every day at 6% of body weight. Feces and uneaten food were removed every day by suction.

According to the maximal detected concentrations in waters and their effective concentrations [5,6], fish were randomly assigned into different treatments, including E2 alone (160 ng/L, T1) and BaP alone (20, 50, or 100 μg/L, T2-T4:) and their combined treatments (T5-T7) with the corresponding concentrations of each chemical in alone treatments. Water and solvent controls with the same amount of dimethylsulfoxide as the exposure treatments at 0.01‰. Six replicate tanks per treatment were conducted with 10 fish in each tank. The exposure were conducted in a continuous-flow exposure system, and 100% of the exposure solution was replaced daily to ensure the stabilization of test chemical concentrations. Water temperature was maintained at 20±1 °C, with pH 7.0±0.2, and dissolved oxygen >95% during the exposure period.

Sample preparation. Fish were sampled at 3, 7, 10 and 14 d and then anaesthetized with MS-222 (J&K Chemical, Shanghai, China). Blood samples (about 1 mL) were collected via a heparinized syringe from the caudal peduncle and immediately centrifuged for 10 min at 10000 g and 4 °C. The supernatants were collected for serum Vtg and E2 analysis. The collected liver samples were homogenized in ice-cold buffer (1:9 w/v, 0.15 mol/L KCl, 0.1 mol/L Tris-HCl, pH 7.4), and then the supernatants were collected for liver Vtg and EROD activity determination after centrifugation for 25 min (10000 g) at 4 °C.

Biochemical assay. The production of Vtg were measured using a Vtg enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA) according to the manufacturer's instructions, which were further normalized to total protein per sample. A diagnostic ELISA kit supported by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) was used to determine the serum E2 levels following the manufacturer's instructions. EROD activity was determined using a fluorescence kit according to the manufacturer's instructions (Genmed Scientifics Inc., Shanghai, China) at the wavelength of 530 nm excitation and 590 nm emission. Protein content in each sample was determined using a BCA protein assay kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), following the manufacturer’s instruction.

Statistical analysis. Statistical analysis was performed with SPSS Statistics 17.0. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison was used to assess the significant differences between different treatments. The differences were considered to be statistical significant when $P<0.05$.

Results

No mortality or deformities were observed in any treatments during the exposure periods. All biomarkers in fish exposed to solvent were not different from those in the water controls, and so the biomarkers were compared with the solvent controls.

Significant increase in the liver and serum Vtg levels were induced by E2, while no Vtg induction was observed in the control and BaP alone treatments (Fig. 1). However, the E2-induced Vtg production both in the liver and serum were all depressed by BaP at lower concentrations, with a 20-25% decreases in the lowest BaP concentration treatment. However, no significant differences of Vtg levels were observed in fish exposed to E2 alone and in combination with the highest

concentration of BaP. For each treatment, more Vtg was produced with the increasing exposure periods and time dependence was apparent. In all cases, more Vtg productions were observed in the liver than those in the serum.

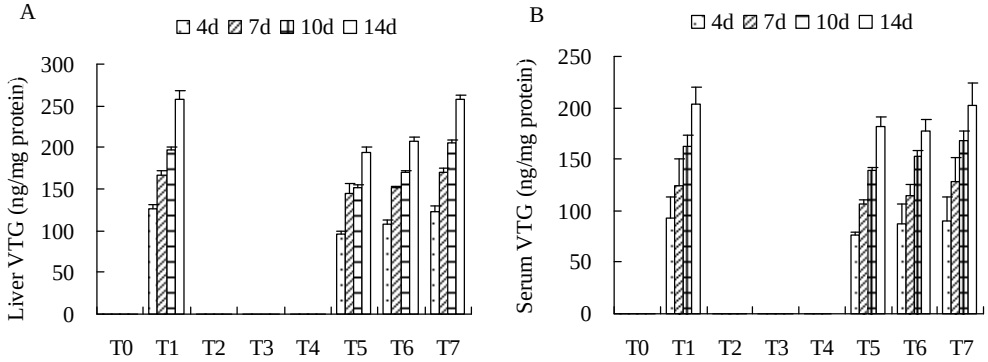

Fig. 1 Vtg level in the liver (A) and serum (B) of male goldfish exposed to the control, E2 and BaP (alone or in combination) (n=15).

Serum E2 levels significantly increased in a time-dependent manner after exposure to E2 alone, however, which were further decreased by the coexistent BaP, especially at low concentration (Fig. 2). No significant differences in serum E2 levels were observed in BaP treatments and the controls.

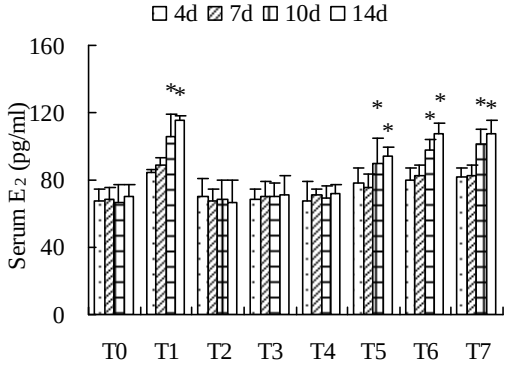

Fig. 2 Serum E2 level in male goldfish exposed to the control, E2 and BaP (alone or in combination) (n=15). Asterisks indicate values that are significantly different from the controls ($P < 0.05$).

Moreover, E2 slightly depress EROD activity compared with the controls (Fig. 3). By contrast, BaP significantly increased the EROD activity, which were higher than that in fish co-exposure to BaP and E2. The EROD activity induced by BaP alone and in combination with E2 all exhibited bell-shaped concentration and time-response curves.

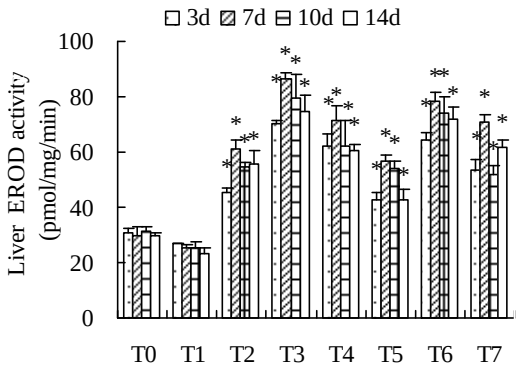

Fig. 3 EROD activity in male goldfish exposed to the control, E2 and BaP (alone or in combination) (n=15). Asterisks indicate values that are significantly different from the controls ($P < 0.05$).

Discussion

Effects of BaP on Vtg levels. The present study shows that the model PAHs, BaP at low concentrations, can reduce the E2-mediated induction of Vtg synthesis in liver and serum of males. Kawahara et al. [7] have demonstrated that CYP1A inducer such as β-naphthoflavone (βNF) and α-naphthoflavone inhibited E1-induced production of Vtg in a concentration-dependent manner on Japanese medaka (*Oryzias latipes*). An *in vitro* study also has shown that exposure to βNF significantly depress the EE2-mediated production of the Vtg in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes [8]. Furthermore, the gene expression of Vtg and *er* in E2-treated fish was also inhibited after co-exposure to βNF [9]. The results of this study are almost in agreement with those previous findings. It therefore indicates that CYP1A inducer is associated with anti-estrogenic effects in teleosts and the estrogenic activity seems to be suppressed by it in the aquatic environment.

Although a number of theories have been used to explain the interaction of estrogens and PAHs on the Vtg production in fish, the mechanism is still unclear. The accelerated metabolism of endogenous E2 observed in serum may account for the decrease in Vtg production after BaP was co-administered with E2. The elevated transcription of biotransformation enzymes, such as EROD activated by BaP, can increase the metabolic rate of circulating estrogens and thereafter indirectly decrease Vtg transcription, as demonstrated by Takemoto et al. [10].

Effects of E2 on EROD activity. In the present study, an inhibiting effect of the CYP1A activity by E2 was confirmed when BaP was co-administered with E2, with a pronounced reduction of EROD activity in liver. Similarly, several studies have demonstrated the ability of estrogens to act as a potent inhibitor of the hepatic EROD activity. A significant decrease of liver EROD activity was observed in immature gilthead seabream (*Sparus aurata L.*) and European flounder (*Platichthys flesus*) treated with E2 [11,12]. Furthermore, Elskus [13] reported that the EROD activity in rainbow trout primary hepatocytes treated with E2 were 15-fold lower than the controls. The mechanism by which estrogens suppress CYP1A expression is not well understood. The suppression can be achieved either by direct or indirect competitive or noncompetitive interaction of the chemical by binding with the enzyme molecule or at the gene transcription level [14].

Cross-talk. The results of the current study demonstrate that E2 can suppress the BaP-mediated increases in EROD activity. At the same time, BaP can reduce the E2-mediated induction of VTG synthesis. Hence, these results suggest that there is a reciprocal inhibiting interaction on biomarker responses in goldfish exposed to E2 and BaP and the two classes of environmental pollutants play an antagonistic effect caused by chemical interactions. This cross-talk on biomarker responses has also been observed in Atlantic salmon (*Salmo salar*) hepatocytes co-exposed to nonylphenol and 3,3’,4,4’-tetrachlorobiphenyl [15].

Conclusions

A reciprocal inhibiting interaction between the estrogens and aromatic hydrocarbons on the established biomarkers, Vtg and EROD activity, in goldfish was observed, and these two classes of environmental pollutants play an antagonistic effect which might cause a decrease in established biomarker responses in fish. The accelerated metabolism of endogenous E2 in serum caused by BaP may account for the decrease in Vtg production induced by E2. More efforts should be made to reevaluate the effects of multiple pollutants and further research to provide more accurate interpretations of the interactions are required.

Acknowledgments

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