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Benzo[a]pyrene modulates the biotransformation, DNA damage and cortisol level of red sea bream challenged with lipopolysaccharide

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ABSTRACT

In animals, biotransformation and the immune system interact with each other, however, knowledge of the toxic mechanism of benzo[a]pyrene (BaP) on these two systems is not well known. The present study investigated the toxic effects of BaP on the biotransformation system, cortisol level and DNA integrity of red sea bream (*Pagrus major*). The results showed that cortisol level was induced under the challenge of lipopolysaccharide (LPS). Short-term exposure (96 h) of BaP at environmental concentration significantly increased the cortisol level, hepatic EROD activity and CYP1A1 mRNA expression. When *P. major* was exposed to BaP for 14 d followed by LPS challenge this increased the cortisol level, EROD activity and hepatic DNA damage except CYP1A1 mRNA expression. Combined with our previous data, which showed that BaP exposure can modulate the immunologic response in *P. major* challenged with LPS, a hypothetical adverse outcome pathway of BaP on fish was suggested.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one class of persistent organic contaminants present in the environment, especially in the marine ecosystem, and they increased mainly due to rapid urbanization and anthropogenic sources (Van Metre and Mahler, 2003, 2005). Benzo[a]pyrene (BaP) is a known genotoxicant which affects both mitochondrial and nuclear DNA and it is also one of the most important carcinogens in the PAH family, which can be activated metabolically by CYP enzymes and epoxide hydrolase to form DNA adducts which exert mutagenic and carcinogenic effects in organisms. BaP can cause oxidative stress by inducing a high level of reactive oxygen species (ROS), which could be related to cellular damage and apoptosis (Bo et al., 2012; Circu and Aw, 2010), and it can also cause embryo toxicity and immunomodulation in both invertebrates and vertebrates (Gopalakrishnan et al., 2009; Wang et al., 2009). Besides its known interference on genotoxicants, it is important to understand the interaction of BaP with other biological and immunological responses.

CYP1A1 is the best studied member of the cytochrome P450 superfamily in fish, and importantly it is known to be induced by dioxins, PAHs and PCBs in the aquatic environment. Moreover, CYP1A1 isozyme activity in the fish liver is used as a biomarker to measure the effect of PAH contaminant (An et al., 2011; dos Anjos et al., 2011). Although CYP1A1 induction is observed in other tissues (Abrahamson et al., 2007; Levine and Oris, 1999; Ortiz-Delgado et al., 2008), the liver was projected to be the most important site of CYP1A1-catalyzed biotransformation in fish due to its roles in the activation and detoxification of foreign compounds. Modulation in hepatic CYPs expression and phase-II enzymes markedly affects the potential risks and benefits of xenobiotics, which is important from a toxicological point of view (Williams et al., 1998).

A previous study reports that corticosteroid hormones can modulate xenobiotic mediated CYP1A responses, and moreover Monostory et al. (2009) suggest in his review that steroidal structure can be AhR ligands and induce CYP1A1 expression in an AhR-dependent manner, and furthermore CYP1A induction response can be potentiated by these hormones and their synthetic derivates in vertebrates, especially in fish and mammals (Celander, 1999; Navas and Segner, 2001; van der Oost et al., 2003). Conversely, the same study also reports both the CYP1A induction process and a baseline suppression of CYP1A protein levels in fish hepatocytes (Celander et al., 1996). Although the increase in stress-dependent plasma corticosteroids influenced by CYP1A expression have been reported, more knowledge is required to understand the effect of stress caused by a contaminant on the CYP1A induction response *in vivo*.

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An earlier study documents that infection can down-regulate biotransformation activities and some studies report that fishes collected from contaminated sites (especially PAH-polluted waters) have more abnormalities in the external region, which are thought to be associated to immunosuppression, including lesions in the skin and fins caused by opportunistic infections (Reynaud and Deschaux, 2006). However, the toxic mechanism by which PAHs induce immunotoxicity in fish remains ambiguous (Bo et al., 2012; Carlson et al., 2004a,b; Reynaud and Deschaux, 2006; Reynaud et al., 2005). In addition, a few studies are reported involving the long term exposure effect of BaP followed by infection.

An adverse outcome pathway (AOP) is a sequence of key events from a molecular-level initiated event and an ensuing cascade of steps to an adverse outcome with population-level significance, which translates mechanistic data to individual-level data centered on key out-comes related to survival and reproduction that can be quantitatively linked to population models (Kramer et al., 2011; Watanabe et al., 2011). Reynaud et al. (2008) consider that the biotransformation pathway may be conserved between mammals and fish, but there is no report on the AOP of BaP in fish.

LPS is the major constituent of the external layer of Gram-negative bacteria and a prototype inducer of inflammation in diverse species. Some research demonstrates that immunomodulating agent LPS has differential effects on fish biotransformation activities (Marionnet et al., 1998; Marionnet et al., 2006), and it is suggested that the effect of this immunostimulant can be mediated through the release of tumor necrosis factor alpha (TNFα) by the immune system (Reynaud et al., 2005). Moreover, our previous results show that the hepcidin gene, an important innate immune component in fish, is significantly induced in fish exposed to an environmentally relevant concentration of BaP (Wang et al., 2009), and BaP has a different modulatory mechanism on the hepcidin gene and other immune associated parameters (Bo et al., 2012), but the modulations of BaP on biotransformation and immune systems in fish remain unknown. This study presents results subsequent to our previous research and the aim was to evaluate the modulation of BaP on the biotransformation system of red sea bream challenged with lipopolysaccharide (LPS). In addition, combination of our present and previous data revealed that BaP exposure modulated an immunologic response in *Pagrus major* challenged with LPS and, as a result, a hypothetical AOP of BaP on fish was suggested.

2. Materials and methods

2.1. Chemicals

BaP (purity, 99%) and LPS (*E. coli* 055:B5) were purchased from the Sigma Company, USA; Power SYBR Green PCR Master Mix from Applied Biosystems, USA; heparin sodium salt, acetone and methanol from Sinopharm Chemical Reagent Co, Ltd., China; and all the chemicals used were of analytical grade.

2.2. Animals

Juvenile *P. major* were obtained from the Zhangpu fish farm, Fujian Province, China. The mean weight and length of fish were 58.94 ± 12.81 g and 12.56 ± 0.96 cm (mean ± SD). The fish were acclimatized to laboratory conditions with a temperature of 12 ± 1 °C, salinity of 30 ± 1‰ and pH 7.8 ± 0.1 for 7 days prior to the experiment in 2000-liter PVC tanks containing seawater treated using sand filtration, kept on a natural daylight cycle and fed with commercial pellets at 1% of BW daily during the acclimation period.

2.2.1. LPS challenge

The fish were challenged with LPS after acclimatization. Briefly, LPS was injected intraperitoneally at a dosage of 5 mg/kg BW in 100 μL of sterile physiological saline solution, and the selected dosage was chosen based on dosage responses in our preliminary experiment (data not shown). The control fish received the same volume of sterile physiological saline solution but LPS-free. Samples ($n = 4$) were collected at 6, 12, 24, 48 and 96 h post LPS-challenge or saline-injection.

2.2.2. Acute exposure of BaP (96 h)

The experiment was carried out for 96 h exposure and the sampling times included 6, 12, 24, 48 and 96 h. Glass aquaria (1 m × 0.5 m × 0.6 m) were used for all the exposure conditions with 12 fish kept in each aquarium. All treatments were performed in duplicate. The concentration of BaP used for the 96 h exposure included 1, 4 and 8 μg/L. The solvent control ($V_{acetone}/V_{seawater}$ = 1/20,000) and a healthy control (blank control) were also maintained. The aqueous BaP concentrations used for the experiment were measured based on the methods of Wang et al. (2009). Briefly, 5 mL water sample was extracted 3 times with 15 mL n-hexane, the extract was concentrated by nitrogen-blowing, then solvent-exchanged into methanol around 1 mL. The BaP concentrations were determined by the fluorescence spectrometry method, and 0.5 mL sample was added to the micro-fluorescence cuvette. The test condition for BaP was ex/em = 380/430, and BaP concentrations were quantified by the standard curve of the standard compound. The seawater and chemicals were renewed daily and the fish were not fed during the exposure period.

2.2.3. Sub-acute exposure of BaP (14 d) followed with LPS challenge

This assay was carried out to assess the immunomodulatory response of fish upon sub-acute exposure of BaP for 14 d. Briefly, *P. major* were first exposed to different concentrations of BaP (1, 4 and 8 μg/L) for 14 d, then moved to fresh seawater either without acetone or challenged with 5 mg/kg LPS for 96 h. Controls, including a normal control group and a solvent control ($V_{acetone}/V_{seawater}$ = 1/20,000) group with sterile physiological saline solution injection, were set up. Parallel duplicate aquaria (1 m × 0.5 m × 0.6 m) were used in the experiments, with 12 fish in each aquarium. The test solution was replaced daily in order to provide a constant effect and the fish were fed daily with commercial pellets at 1% of BW during the exposure period.

2.3. Sampling

Blood samples were collected individually from the caudal vein of fish after each experimental condition (LPS challenge, BaP exposure, and BaP exposure plus LPS challenge). The method for plasma separation was followed as previously described (Thilagam et al., 2009). Briefly, four fish in duplicate were used at each time point. Blood samples were heparinized (50 international units/mL of blood), and the sample was centrifuged at 4500g for 5 min at 4 °C to collect the plasma. At the same time, samples of liver from each individual fish were collected separately and immediately frozen in liquid nitrogen and stored at – 80°C for total RNA extraction.

2.4. Measurement of plasma cortisol level

Plasma cortisol levels were measured using a diagnostic ELISA direct immunoenzymatic kit (Roche, Switzerland). The cortisol in the sample competes with horseradish peroxidase-cortisol for binding onto the limited number of anti-cortisol sites in the microplate. Cortisol concentration in the sample was calculated based on a series of standards and the color intensity was inversely

proportional to the cortisol concentration in the sample. The method allows the determination of cortisol from 10 to 800 ng/mL.

2.5. EROD activity determination in gill and liver

2.5.1. Gill EROD activity

The gill EROD activity was measured based on the methods of Jonsson et al. (2002) with minor modification. Briefly, the gill arches were excised and placed in ice-cold HEPES-Cortland (HC) buffer (0.38 g of KCl, 7.74 g of NaCl, 0.23 g of $MgSO_4{\cdot}7H_2O$, 0.23 g of $CaCl_2{\cdot}2H_2O$, 0.41 g of $NaH_2PO_4{\cdot}H_2O$, 1.43 g of HEPES, and 1 g of glucose per 1 L of dH_2O; pH 7.8). Then, the filaments were cut immediately, resulting in tip pieces about 3 mm long. From each fish, duplicate groups of seven tip pieces were transferred to wells of a culture plate containing HC buffer. Following 10 min of preincubation in reaction buffer (HC buffer supplemented with 10^{-6} M 7-ethoxyresorufin and 10^{-5} M dicumarol), the buffer was replaced with 0.7 mL of fresh reaction buffer. After 10 and 30 min of incubation, 0.2 mL aliquots were transferred from each well to a Fluoronunc 96-well plate. The fluorescence was determined as described below.

2.5.2. Hepatic EROD activity

The hepatic EROD activity was measured as previously described (Thilagam et al., 2010). Briefly, hepatic EROD determination microsomal pellets were resuspended in 500 μL of Tris–HCl buffer. Cytosolic and microsomal protein contents were measured using the method of Bradford, with bovine serum albumin as the standard. In the microsomal fraction, EROD activity was determined using the method described by Burke and Mayer (1974) using a microplate reader at 535/585 nm excitation/emission wavelengths.

2.6. Evaluation of DNA strand breaks

The alkaline unwinding assay used in the study described by Thilagam et al. (2010). Briefly, liver samples were gently cut into fine pieces and incubated with 1 mL of TNE buffer (0.05 M Tris, 0.1 M NaCl, 0.1 M EDTA, 0.5% SDS, pH 8.0) at 37 °C for 24 h. After incubation, saturated NaCl was added and the mixture centrifuged at 12,000*g* for 20 min. The supernatant was added to an equal volume of buffered phenol/chloroform/isoamylalcohol (PCI) (25:24:1, v/v, pH 8.0) and gently mixed. The sample was allowed to settle for 5 min before centrifuging at 12,000*g* at 4 °C for 5 min. The transferred aqueous layer was then digested using 5 μL of Ribonuclease A (10 mg/mL) for 30 min at 37 °C and extracted successively using equal volumes of PCI. The DNA was precipitated by adding cold absolute ethanol and sodium acetate buffered to pH 5.2. The sample was centrifuged at 12,000*g* for 15 min, and the pellet was rinsed with 500 μL of 70% ethanol, air dried and then redissolved in 400 μL of TE buffer (10 mM Tris, 1 mM EDTA). The DNA sample was separated into two equal portions for fluorescence determination of double stranded DNA (dsDNA) and single-stranded DNA (ssDNA). The fluorescence of dsDNA and ssDNA was measured using a spectrofluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The DNA strand break was calculated as *F* values, determined by dividing the double strand value by the double plus single strand value in the sample.

2.7. Quantitative real-time PCR (qPCR) analysis of the CYP1A1 mRNA expression

Total RNA was extracted from the liver of fish under different treatments using the TRIzol method (Invitrogen), and then reversely transcribed into cDNA using the One-Step TaKaRa Primescript™ RT Reagent Kit (Perfect Real-time, TaKaRa). 18S rRNA was used as the endogenous control gene, and the gene specific primers for qPCR (18S rRNA and CYP1A1) of *P. major* were the same as in our previous reports (Bo et al., 2012; Wang et al., 2009). The qPCR assay was carried out as previously described. Briefly, qPCR assays were performed using the fluorescent dye Power SYBR Green PCR Master Mix and an ABI 7500 System, and the expression levels of the tested genes were calculated using the relative expression software tool (ABI), based on the $2^{-\Delta\Delta CT}$ relative response method (Livak and Schmittgen, 2001). The mRNA expression level of *P. major* CYP1A1 (PM-CYP1A)1 in liver for the normal fish (without any treatment) was taken as a calibrator, and all the data of fold change were obtained in comparison with the same calibrator.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 17.0. Results are reported as mean ± SD. The data were first tested for normality and homogeneity using Bartlett's test. ANOVA followed by Tukey's multiple comparison post hoc-test was performed to identify statistical differences between group means. Dunnett test was performed to see the difference within the group and the differences were given in the figure using asterisks. Pearson correlation matrix analyses were used to test whether there was any interaction among the measured parameters. Since there was no significant difference between the solvent and blank control, we show only the results of the solvent control in the figures.

3. Results

3.1. Plasma cortisol level

The changes in the level of plasma cortisol concentration are shown in Fig. 1. The cortisol level showed no change in *P. major* after intraperitoneal injection of 5 mg/kg LPS from 6 to 12 h, but a significant increase was observed at 24 and 48 h post challenge compared to the respective control group (Fig. 1A). During the acute exposure, plasma cortisol levels increased in the fish exposed to the lowest concentration (1 μg/L) of BaP after 6, 24 and 96 h; and moreover the results showed a significant increase after 24 h in fish exposed to 4 and 8 μg/L of BaP (Fig. 1B), however no significant difference between the time points ($p < 0.570$) was observed. The sub-acute effects of BaP exposure followed by 5 mg/kg LPS challenge on cortisol level of *P. major* are shown in Fig. 1C. Similar to the acute exposure results, cortisol levels were elevated in the fish exposed to the lowest concentration (1 μg/L) of BaP followed by the injection of LPS from 6 to 48 h, but there was no significant increase after 96 h post challenge compared to the respective control group (Fig. 1C), similarly there was no significant differences observed between the time points ($p < 0.710$).

3.2. EROD activities

The gill EROD activities in the different groups are presented in Fig. 2. The LPS injection showed no significant modulation of EROD activity (Fig. 2A). Acute BaP exposure resulted in a significant induction of gill EROD activities ($p < 0.05$) after 48 and 96 h in both the higher concentrations, but conversely the lowest concentration (1 μg/L) and the highest concentration (8 μg/L) significantly decreased the EROD activity after 6 h of BaP exposure (Fig. 2B). However no significant difference between the time points ($p < 0.701$) was observed. Sub-acute exposure followed by LPS challenge induced EROD activity in *P. major*, when the fish were exposed to 8 μg/L of BaP followed by the LPS challenge induced EROD activity from 6 to 96 h, however the other concentrations

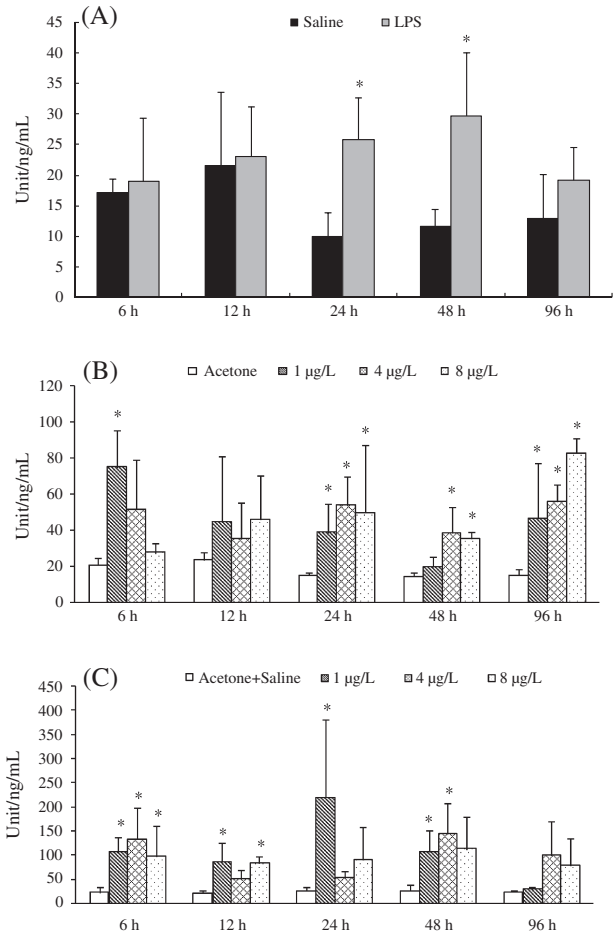

Fig. 1. Cortisol level of juvenile *P. major* exposed to different concentrations of BaP following challenge with LPS. (A) LPS challenge, (B) acute exposure of BaP and (C) sub-acute exposure of BaP followed by LPS challenge. An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

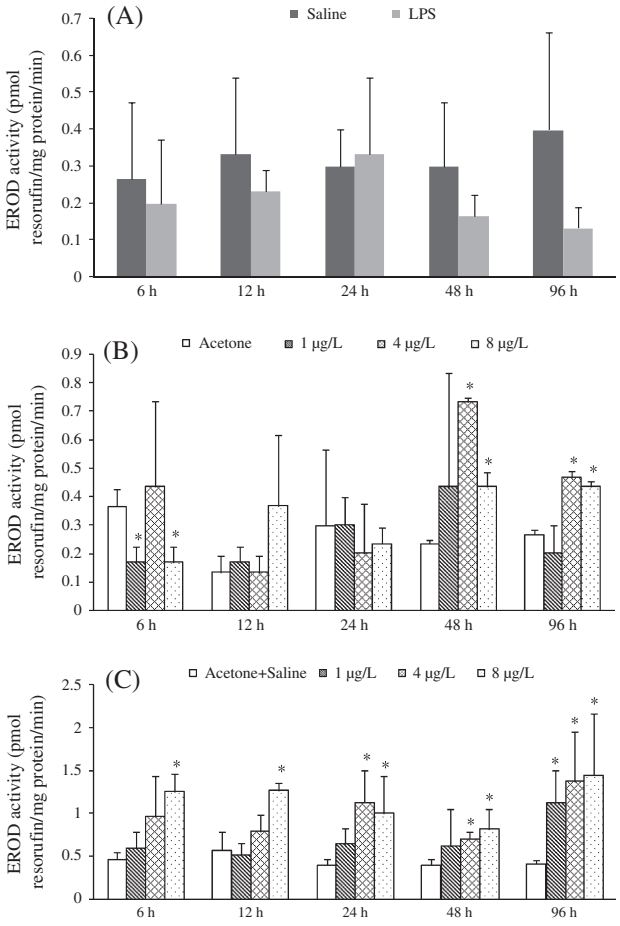

Fig. 2. The liver EROD activity of juvenile *P. major* exposed to different concentrations of BaP following challenge with LPS. (A) LPS challenge, (B) acute exposure of BaP and (C) sub-acute exposure of BaP followed by LPS challenge. An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

showed significant increase only after 24 h post challenge of LPS (Fig. 2C) and when compared between the time points no significant difference was observed ($p < 0.780$).

Similar to gill EROD activity, the hepatic EROD activity showed no significant modulation after LPS challenge (Fig. 3A), whereas the acute BaP exposure induced a significant induction of hepatic EROD activities from 6 to 96 h in the two higher concentrations. In addition, the lowest concentration of BaP significantly increased the EROD activity after 6 h of BaP exposure (Fig. 3B). Similarly the sub-acute exposure followed by LPS challenge induced EROD activity in *P. major*, when the fish was exposed to both of the higher concentrations of BaP from 6 to 96 h (Fig. 3C), however there was no significant differences between the time points ($p < 0.136$).

3.3. CYP1A1 mRNA expression

The transcriptional profiles of the PM-CYP1A1 gene in the liver of *P. major* were determined using qPCR (Fig. 4). When the fish were exposed to different concentrations of BaP (1, 4 and 8 μg/L), the dosage- and time-dependent transcriptional patterns were observed (Fig. 4A). No significant change of PM-CYP1A1 was observed from 6 h to 24 h after the fish exposed to BaP at a dosage of 1 μg/L, and it was remarkably induced to 17-fold at 48 h ($p < 0.05$). A significant increase of PM-CYP1A1 mRNA expression occurred at 6 h, 12 h and 48 h, and this increased to around 12-fold, 7-fold and 63-fold ($p < 0.05$), respectively, at a dosage of 4 μg/L. For the highest concentration of BaP (8 μg/L) exposure group, transcription of PM-CYP1A1 was significantly up-regulated at all time-points from 6 h to 48 h, and reached a peak at 24 h (48-fold), and then it gradually decreased to 13-fold ($p < 0.05$). In particular, the transcription of PM-CYP1A1 decreased to basal level at 96 h for all three BaP exposure group concentrations (Fig. 4A), though the modulation of PM-CYP1A1 expression after BaP exposure, there was no significant differences observed between the time points ($p < 0.250$). Compared with the acute exposure of BaP, no significant change was observed during the whole experimental period when *P. major* was exposed to the different concentrations of BaP followed by the 96 h LPS challenge (Fig. 4B).

3.4. DNA strand breakage

DNA strand breakage in the different groups is presented in Fig. 5. There was no difference in DNA strand breakage when the fish was challenged with LPS alone (Fig. 5A). DNA strand breaks in *P. major* liver were observed after acute and sub-acute exposure to BaP. With acute exposure, both the high concentrations groups exhibited decreased *F* values after 12 h (Fig. 5B). Similarly, sub-acute exposure of *P. major* to the higher BaP concentrations followed by LPS challenge showed significant difference in *F* values with the respective control after 12 h, and there was a significant decrease from 6 h to 24 h in almost all the groups (Fig. 5C).

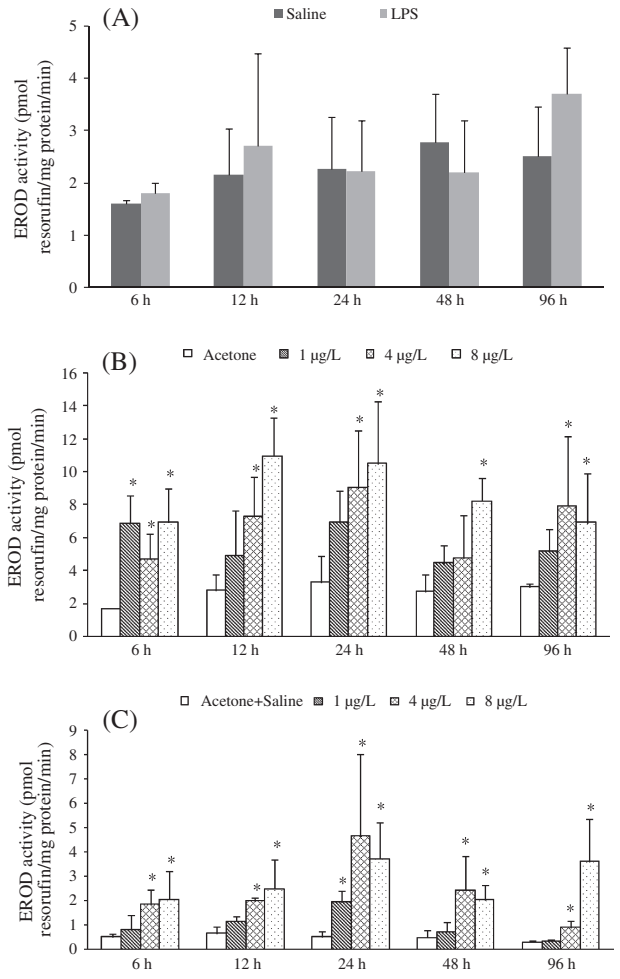

Fig. 3. The gill EROD activity of juvenile *P. major* exposed to different concentrations of BaP following challenge with LPS. (A) LPS challenge, (B) acute BaP exposure and (C) sub-acute exposure of BaP followed by LPS challenge. An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

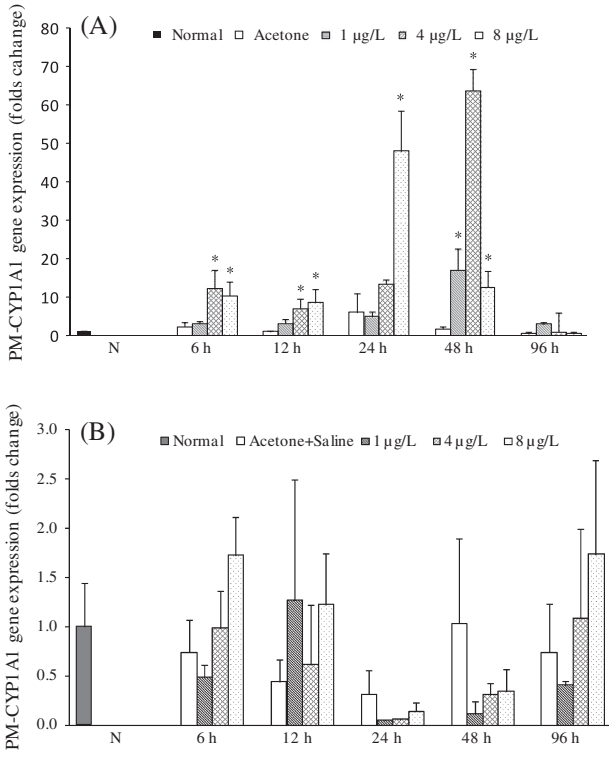

Fig. 4. CYP1A1 mRNA expression in the liver of juvenile *P. major* exposed to different concentrations of BaP followed by LPS challenge. (A) Acute exposure of BaP, and (B) sub-acute exposure of BaP followed by LPS challenge. Asterisks (*, **) indicate statistical significance at $p < 0.05$ or $p < 0.01$ between the treatments and the corresponding controls.

3.5. Correlation analysis for the measured parameters

The Pearson correlation matrix calculated is given in Table 1. The correlation among the biomarkers produced similar results for both acute and sub-acute exposure of BaP to *P. major*. There was no significant correlation observed between the parameters when the fish were challenged with LPS alone, except between DNA damage and cortisol level. However, during acute BaP exposure significant correlation existed between hepatic DNA damage and EROD activities in both the hepatic and gill tissues. Similarly, the gill and hepatic EROD activities showed a good significant relationship with CYP1A1 mRNA expression. In 14 d sub-acute exposure of BaP to *P. major* followed by LPS challenge, cortisol and hepatic EROD activity showed good correlation between them. Further we also observed a significant relationship between DNA damage and hepatic EROD activity. The correlation between CYP1A1 mRNA expression with DNA damage and hepatic EROD activity was high. Although there was a relationship observed between cortisol and DNA damage it showed no statistical significance.

4. Discussion

In seawater BaP may exist in three states: completely dissolved, adsorbed on the suspended particles, or as colloidal material (Kucklick and Bidleman, 1994), and moreover the solubility of BaP in water is very low (3.8 μg/L at 25 °C) due to its hydrophobic nature (Manoli and Samara, 1999). PAHs associate easily with particulate matter, especially particulate matter with higher organic carbon contents, and are finally deposited in the sediment, although the concentration of BaP in pore water is much higher (12.2–96.8 μg/L) from the Jiulong River Estuary and Western Xiamen Sea, China. However the surface water shows a range between 0.56 and 3.32 μg/L in the same region (Maskaoui et al., 2002) and hence environmentally relevant concentrations of BaP were used for prolonged waterborne exposure.

The results of our present study revealed that LPS does not have much influence on the cortisol level, however both acute and sub-acute exposure of BaP increased the plasma cortisol. The influence of PAHs is reported to increase the cortisol level in fish and our study was consistent with the earlier studies which show that injection of PAHs elicits an increase in plasma cortisol (Aluru and Vijayan, 2004; Gesto et al., 2008; Tintos et al., 2008). However, the sub-acute exposure to BaP followed by the LPS challenge initially showed the peak cortisol level and it was reduced significantly during the course of the experiment. Moreover it is documented that plasma cortisol falls back to the resting levels during chronic stress, even though the fish may still be responding to the stressor (Vijayan and Leatherland, 1990).

LPS is the major constituent of the external layer of Gram-negative bacteria and a prototype inducer of inflammation in diverse species. Some research demonstrates that immunomodulating agent LPS has differential effects on fish biotransformation activities (Marionnet et al., 1998, 2006), and it is suggested that the effect of this immunostimulant can be mediated through the release of TNFα by the immune system (Reynaud et al., 2005). In our study, hepatic and gill EROD activities were not significantly

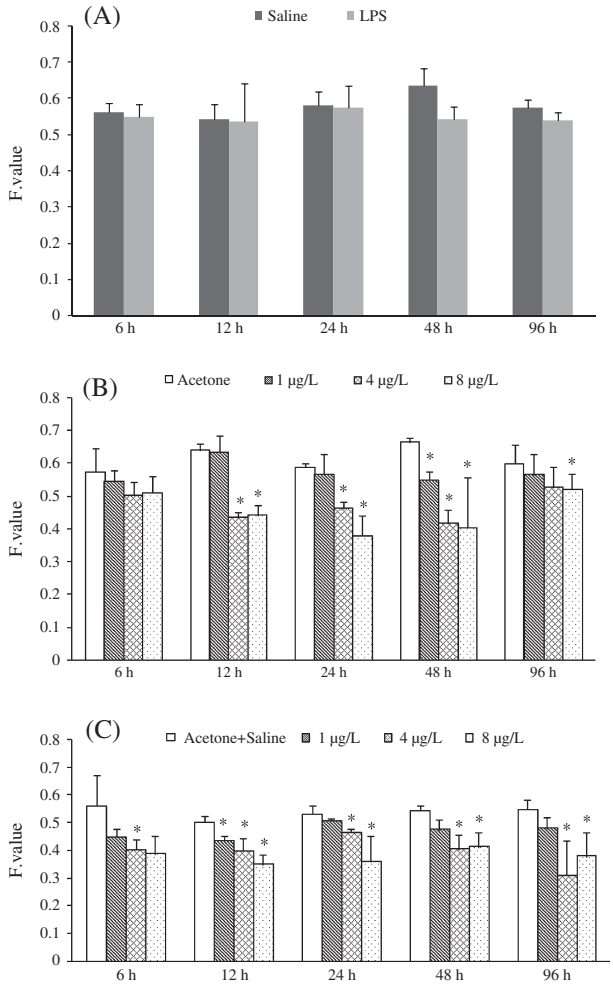

Fig. 5. The DNA damage in the liver of juvenile *P. major* exposed to different concentrations of BaP following challenge with LPS. (A) LPS challenge, (B) acute exposure of BaP and (C) sub-acute exposure of BaP followed by LPS challenge. An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

modified following challenge with LPS (Figs. 2A and 3A). This phenomenon may suggest that, both in mammals and fish, the capacity of LPS to regulate biotransformation activities is organ specific and may imply different mechanisms (Reynaud et al., 2008).

Gill EROD activity is a more sensitive biomarker of exposure to waterborne CYP1A inducers than EROD activities in liver and kidney (Abrahamson et al., 2007). On the contrary, the results of our present study showed that hepatic EROD activity was more sensitive than gill EROD activity, and the induction of hepatic EROD activity significantly increased in *P. major* exposed to a higher dosage of BaP (4, 8 μg/L) from 6 h to 96 h (Fig. 3B). However, gill EROD activity was significantly induced only after 48 h and 96 h for the same dosages, and the hepatic EROD activity was about ten-fold higher than that in gill, and furthermore the hepatic EROD activity was significantly induced for 14 d sub-acute BaP exposure (Fig. 2B). Hence, we may conclude that hepatic EROD activity was more sensitive than gill EROD activity to BaP waterborne exposure.

BaP induced cytochrome P450 proteins via an intracellular aryl hydrocarbon receptor (AhR) is reported. Upon binding of the BaP, the receptor translocates to the nucleus where it dimerizes with tRNA (AhR nuclear translocator). The AhR–tRNA heterodimer binds to specific xenobiotic responsive elements (XRE) present in certain CYP genes which can induce later expression (Reynaud and Deschaux, 2006). Based on our previous study both CYP1A1 and AhR2 gene expression are quickly induced and then gradually return to the basal levels with BaP exposure, and there is a time- and dose-dependent response of these two genes at transcription level (Bo et al., 2010). The interaction between immune and biotransformation systems is more complicated, and the regulation mechanism is not fully understood yet and so it is important to know the AOP of BaP. Based on our present results and existing work on BaP toxicity we drew a pathway which could produce an adverse effect due to BaP toxicity (Fig. 6).

Immunostimulants have been shown to suppress P450-dependent drug biotransformation. Further, immunostimulants can directly or indirectly activate macrophages via macrophage activating factor secretion or Kupffer cells leading to the generation of ROS, which is the important mechanism for the protection of a host against pathogens and also for repairing its own damaged tissues (Thilagam et al., 2009). However, generation of excessive superoxide anions can destroy some molecules at the mRNA level (Reynaud et al., 2008). In our previous study we report that ROS production is significantly induced and maintained for a long period when *P. major* is exposed to environmentally relevant concentrations of BaP (1, 4 and 8 μg/L) followed by LPS challenge (Bo et al., 2012), which can ultimately lead to DNA damage and CYP mRNA destruction (Figs. 4B and 5). We also speculate that one of the other parts of BaP induced immunotoxicity is mediated through the production of BaP metabolites by cytochrome P450 or related enzymes (Carlson et al., 2004b; Reynaud et al., 2008).

Table 1
Correlation matrix for the measured parameters in red sea bream exposed to (A) LPS, (B) BaP and (C) BaP + LPS.

		Cortisol	DNA damage	EROD gill	EROD liver	Cyp1a1 expression
LPS	Cortisol	1.000	−0.626[a]	−0.412	−0.100	–
	DNA damage		1.000	0.485	−0.21	–
	EROD gill			1.000	−0.295	–
	EROD liver				1.000	–
	Cyp1a1 expression					1.000
BAP	Cortisol	1.000	0.400[a]	0.370	0.182	−0.123
	DNA damage		1.000	−0.782[b]	−0.528[b]	−0.354
	EROD gill			1.000	0.586[b]	0.410[a]
	EROD liver				1.000	−0.009
	Cyp1a1 expression					1.000
LPS + BaP	Cortisol	1.000	−0.358	0.084	0.582[b]	−0.024
	DNA damage		1.000	−0.361	−0.745[b]	−0.630[b]
	EROD gill			1.000	−0.016	0.399[a]
	EROD liver				1.000	0.390[a]
	Cyp1a1 expression					1.000

"–" Indicates not measurement.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

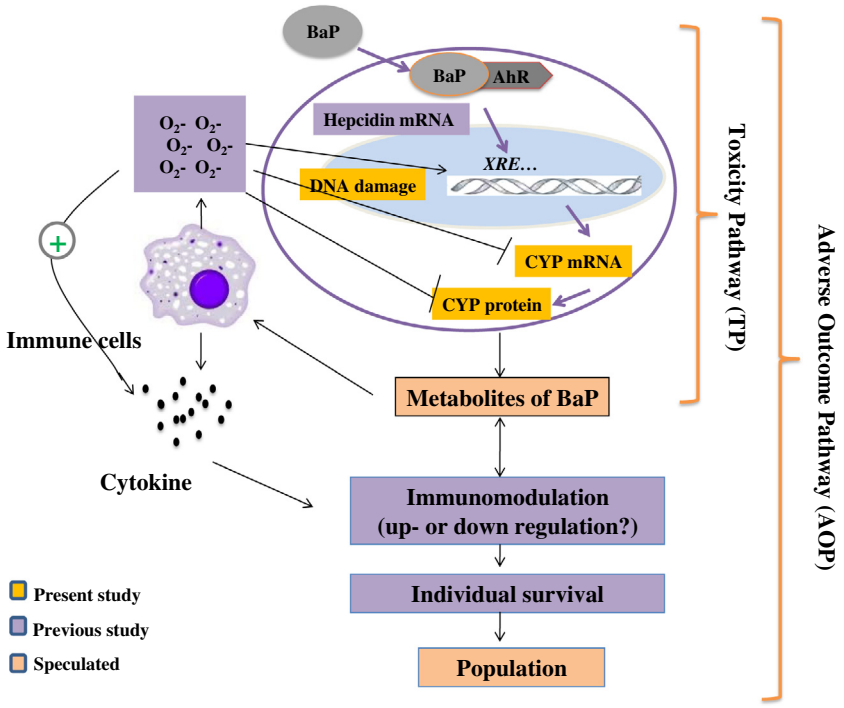

Fig. 6. The speculated adverse outcome pathway (AOP) model of BaP in fish.

Immunomodulation due to BaP leads to either up- or down - regulation of the immune system. Previous studies report that BaP exposure can suppress the immune function in Japanese medaka (*Oryzias latipes*) and reduce the host resistance against bacterial challenge (Carlson et al., 2002, 2004a,b). On the contrary, BaP enhances some immune associated parameters in *P. major* and in *Mytilus edulis* (Bo et al., 2012; Brown et al., 2006; Wang et al., 2009). Similarly, a few other studies report that environmental contaminants such as pesticides and polybrominated diphenyl ethers decrease the disease susceptibility of *Oncorhynchus mykiss* and *Oncorhynchus tshawytscha* (Arkoosh et al., 2010; Shelley et al., 2009) but whether or not this immunomodulation (suppression or expression) would help the organism to survive a future infection remains to be elucidated. However, based on earlier reports, undoubtedly the regulation of BaP of the host resistance to bacterial challenge will ultimately affect individual mortality and the fish population (Wang et al. 2009; Bo et al., 2012).

In this study, the PM-CYP1A1 mRNA expression pattern was modulated and the results were completely different from those with BaP exposure alone, when compared with juveniles of *P. major* exposed for 14 d followed by LPS challenge, and this change of PM-CYP1A1 mRNA expression might have been due to the higher level of ROS generated due to the additive effect of BaP and LPS. Thus, we concluded that the modulation of environmentally relevant concentrations of BaP on biotransformation and immune systems can affect the host resistance to bacterial challenge, and this will ultimately affect individual mortality and even fish populations.

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