Benzo[*a*]pyrene Modulation of Acute Immunologic Responses in Red Sea Bream Pretreated with Lipopolysaccharide

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ABSTRACT: The effects of polycyclic aromatic hydrocarbons (PAHs) have been reported to modulate the immune response in aquatic animals, but the collected information of their effects on fish immunity is so far ambiguous. This study demonstrated that Benzo[*a*]pyrene (BaP) exposure altered the expression pattern of an antimicrobial peptide hepcidin (PM-hepc) gene and the activities of some immune-associated parameters in the lipopolysaccharide (LPS)-challenged red sea bream (*Pagrus major*). It was observed that LPS could increase respiratory burst, lysozyme and antibacterial activity in *P. major*. However when the *P. major* was exposed to different concentrations of BaP (1, 4, or 8 μg L^{-1}) for 14 days and then challenged with LPS there was no significant change in the lysozyme and antibacterial activity. It was further observed that LPS could induce the PM-hepc mRNA expression at 3, 6, and 12-h post-LPS challenge. However, when *P. major* was exposed first to BaP for 14 days and then challenged with LPS, the expression of PM-hepc mRNA was delayed in the liver until 24 h and not significantly induced until 48 and 96 h. The mRNA expression pattern was completely different from that only with LPS challenge, showing that BaP exposure changed the PM-hepc mRNA expression pattern of fish with LPS challenge. This study demonstrated that BaP exposure can weaken or inhibit the induction of lysozyme and antibacterial activity in the LPS-challenged *P. major*; conversely BaP exposure could enhance the mRNA expression of PM-hepc gene, indicating that the effect of BaP has different modulatory mechanism on hepcidin genes and immune-associated parameters. © 2012 Wiley Periodicals, Inc. Environ Toxicol 29: 517–525, 2014.

Keywords: *Pagrus major*; benzo[*a*]pyrene; immunomodulation; lipopolysaccharide; hepcidin

INTRODUCTION

The teleosts possess innate and acquired immunity even if they belong to the most primitive vertebrates. The teleosts mainly depend on the innate immune system because the acquired immune system is not perfectly developed. In higher vertebrates the immune system show high sensitivity to the xenobiotics present in the environment (Inadera, 2006) and the host immune system establishes different defensive means against the exogenous hazard such as induced production of antimicrobial peptide and generation of reactive oxygen species (ROS). It is worthwhile to note that ROS-mediated antimicrobial activity is powerful in host defense system but overproduction of ROS could be deleterious to the host homeostasis, thus its production must be tightly controlled by the host (Jiang et al., 2007; Lushchak, 2011). Though the studies revealed hemocyanin and hemoglobin could produce ROS in both invertebrates

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and vertebrates but how it is regulated by the microbial virulence factors during infection and the importance of ROS-mediated antibacterial activity are not fully understood in both cases. The evidence from earlier studies show that the immune function is apparently modulated before the target organs, tissues or effective cells show any visible toxicity or histochemical changes and its endpoints appear to be more sensitive than the hepatic CYP450 activity measured in deer mice (*Peromyscus maniculatus*) exposed to polycyclic aromatic hydrocarbons (PAHs) (Peden-Adams et al., 2007). Studies on immunotoxicological risk assessment using rodents and other mammalian species revealed that the innate immune response against pathogens may be more impacted by xenobiotic mediated suppression in comparison with the acquired immune response (Luster et al., 1988).

Benzo[*a*]pyrene (BaP) is a five-ring PAH usually formed by the combustion of organic material. This environmental xenobiotic widely exists in air, water, and sediment, (Carlson et al., 2002). The immune system of fish is very sensitive to PAHs, and these pollutants affect both innate and acquired immunity (Reynaud and Deschaux, 2006). Exposure to PAHs is reported to modulate the immune response in aquatic animals, although the effect is different due to the mode of exposure, the dosage used or the species studied. The lysozyme levels are suppressed in rainbow trout (*Oncorhynchus mykiss*) injected with an oil-based drilling mud extract after 6 weeks and also the serum lysozyme activity is decreased in dab (*Limanda limanda* L.) exposed to oil-contaminated sediments for 2–4 weeks (Tahir and Secombes, 1995; Secombes et al., 1997). Similarly, BaP exposure at sublethal concentration significantly modulates the activities of some immune associated parameters in abalone (*Haliotis diversicolor*), (Gopalakrishnan et al., 2009), and BaP exposure suppresses the immune function of Japanese medaka (*Oryzias latipes*) and reduces its resistance to bacterial challenge (Carlson et al., 2002; Carlson et al., 2004a,b). However, the effects of pollutant exposure on mRNA expression in fish are complex. Some immune-associated genes are downregulated after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or oil, but conversely some genes (such as interleukin-8 and lysozyme), which are involved in proinflammatory processes, are upregulated upon oil exposure (Volz et al., 2005; Nakayama et al., 2008). Similarly, an antimicrobial peptide hepcidin gene is downregulated in largemouth bass (*Micropterus salmoides*) exposed to an endocrine disrupting compound 17-β estradiol (Robertson et al., 2009), but it is upregulated in the developmental stages of red sea bream *Pagrus major* upon exposure to BaP (Wang et al., 2009a). Understanding of the interaction between innate immunity and toxicant effects should improve the ecotoxicological goal of well safeguarding fish populations and even the aquatic ecosystem health (Bols et al., 2001). Indeed, the measurement of immunological performance is suggested as an important criterion in the determination of stressor or toxicant exposure in fish (Kennedy and Farrell, 2008). In addition, a growing interest in elucidating the effects of environmental contaminants on the immune system has been stirred up with the increased observation of pollution induced disease conditions in marine organisms (Pipe and Coles, 1995; Carlson et al., 2002; Song et al., 2008).

Our previous study revealed that the short-term exposure of BaP significantly induced hepcidin genes expression (PM-hepc and AS-hepc2) in the developmental stages of *P. major* and in juvenile *Acanthopagrus schlegelii* B. (Wang et al., 2009a). To further understand the interaction between the toxic effect of BaP and the immunomodulation of fish hepcidin expression in the juvenile stage of *P. major*, the present work continued to investigate the prolonged exposure of BaP on the mRNA expression pattern of PM-hepc gene after lipopolysaccharide (LPS) challenge. In addition, the activities of some immune-associated parameters such as respiratory burst, antibacterial, and lysozyme activity were measured in parallel in the blood component of *P. major* after acute and subacute exposure to BaP at an environmental relevant concentration. This study will further present evidence to strengthen the previous observations that the environmental pollutant BaP could alter the gene expression pattern or activities of some immune associated components and would provide insightful information on elucidating the immunomodulatory mechanism of BaP effect on fish immunity.

MATERIALS AND METHODS

Chemicals

Benzo[*a*]pyrene (purity, 99%), nitro blue tetrazolium (NBT), LPS (*E. coli* 055:B5), hen egg white lysozyme (HEWL) and *Micrococcus lysodeikticus* were purchased from Sigma (Sigma Chemicals, St. Louis, MO). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems, USA. Heparin sodium salt, acetone and methanol were all purchased from Sinopharm Chemical Reagent, China and all the chemicals used were of analytical grade.

Animals

Juvenile *P. major* were obtained from 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 (means ± SD). The fish were acclimatized to laboratory conditions with a temperature of 12°C ± 1°C, salinity of 30‰ ± 1‰ and pH 7.8 ± 0.1 for 7 days prior to the experiment in 2000-L PVC tanks containing seawater treated using sand filtration, kept on a natural daylight cycle and fed with commercial pellets at 1% of body weight (BW) daily during the acclimation period.

LPS Challenge

The fish were challenged with LPS after acclimatization. Briefly, LPS was injected intraperitoneally at a dosage of 5 mg kg^{-1} BW in 100 μL of sterile physiological saline solution, and this 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. The samples (n = 3–4) were collected at 3, 6, 12, 24, 48, and 96-h post-LPS challenge or saline-injection.

Acute Exposure of BaP (96 h)

The experiment was carried out for 96-h exposure and the sampling times included 3, 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 96-h exposure includes 1, 4, and 8 μg L^{-1}, respectively. Solvent control ($V_{acetone}/V_{seawater}$ = 1/20 000) and healthy control (blank control) were also maintained. BaP concentrations used for the experiment were measured during the exposure period using Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, USA). The seawater and chemicals were renewed daily and the fish were not fed during the exposure period.

Subacute Exposure of BaP (14 Days) Followed with LPS Challenge

This assay was carried out to assess the immunomodulatory response of fish upon subacute exposure of BaP for 14 days. Briefly, *P. major* were first exposed to different concentrations of BaP (1, 4, and 8 μg L^{-1}) for 14 days, then moved to fresh seawater without acetone and challenged with 5 mg kg^{-1} LPS for 96 h, respectively. The controls including a normal control group, 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.

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 serum separation was followed as previously described (Thilagam et al., 2009). Briefly, three to four fish in duplicate were used at each time point. Blood samples were divided into two aliquots, one was heparinized (50 international units (IU) per mL of blood), and the other allowed to clot at room temperature for 30 min and then kept at 4°C for 24 h. The clotted sample was centrifuged at 4500 × g for 5 min at 4°C to collect the serum, which was stored immediately at −80°C until use. The heparinized blood was used to measure the respiratory burst activity in all treatment groups. 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.

Serum Lysozyme Assay

The activity of lysozyme was measured as previously described (Thilagam et al., 2009). Briefly, a 0.3 mg mL^{-1} suspension of freeze-dried *M. lysodeikticus* was prepared in 0.05 M Na_2HPO_4 buffer immediately before use and the pH was adjusted to 6.0 using a few drops of 1.0 M NaOH. Ten μL of serum were added to 250 μL of the bacterial suspension and allowed to equilibrate at 28°C. Hen egg white lysozyme with a specified activity of 46 200 U mg^{-1}, was used as an external standard. After dissolving the HEWL in buffer at a concentration of 25 000 U mL^{-1}, this HEWL solution was added to each microplate well (10 μL $well^{-1}$). The reduction in absorbance at 450 nm was determined over a 10-min period at 28°C in a microplate reader. The standard curve was constructed using HEWL. The lysozyme activity of the serum was calculated from this standard curve.

Antibacterial Activity of Serum

The antibacterial activity of the serum was investigated by measuring the growth inhibition with turbidometry, using the methods previously described (Gopalakrishnan et al., 2009). Briefly, 100 μL of serum from both the control and experimental groups was added to a 96-well plate. A log phase broth culture of *Aeromonas hydrophilla* was grown overnight in nutrient broth (NB) with constant shaking at 28°C. A bacterial suspension (100 μL) in NB was prepared (~10^8 bacteria per mL; OD_{600} = 0.509) and added to each of the experimental and control wells. Aliquots of 100 μL sterile phosphate buffer solution (PBS) and 100 μL sterile NB were also added to a well to act as a blank. The plate was incubated at room temperature and absorbance measured after 0, 1, and 24 h at 540 nm.

Respiratory Burst Activity

The procedure for intracellular superoxide anion production was followed as described in our previous study (Thilagam et al., 2009). Briefly, 50 μL of heparinized blood was

placed into a tube and incubated at 37°C for 1 h, and then the cells which had adhered were washed with PBS (pH 7.2). After washing, 50 μL of 0.2% NBT was added and the cells were incubated for a further 1 h. The tubes were centrifuged at 600 × g for 10 min and the supernatant was removed. The cells were fixed with 100% methanol for 2–3 min. The tubes were then air-dried and 2 mL of extraction fluid (6 mL KOH and 7 mL dimethyl sulfoxide) was added into each tube and they were then centrifuged at 7000 × g for 15 min. The OD of the supernatant was read at 595 nm against the reagent blank in a spectrophotometer.

Quantitative Real-Time PCR (qPCR) Analysis of the Hepcidin mRNA Expression

Total RNA was extracted from the livers of fish under different treatments using the TRIzol method (Invitrogen), and then reversely transcribed into cDNA using the One-Step TaKaRa Primescript^{TM} RT Reagent Kit (Perfect Real-time, TaKaRa). The 18S rRNA was used as the reference gene, and the genes specific primers (18S rRNA and hepcidin) of *P. major* were the same as the previous report (Wang et al., 2009a). The amplification efficiency of the PM-hepc and the endogenous control 18S rRNA gene were verified, which were approximately equal in qPCR assay. The qPCR was carried out as previously described (Wang et al., 2009a). Briefly, qPCR assays were performed using the fluorescent dye Power SYBR Green PCR Master Mix and an ABI 7500 System, and the relative 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 PM-hepc 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.

Statistical Analysis

Statistical comparisons were performed using a two-way analysis of variance (ANOVA) and SPSS software (Ver 17.0; SPSS). Results are reported as mean ± S.D. of four individuals per group per time point and the significance was tested. The data were first tested for normality and homogeneity using Bartlett's test. Because all data were normal, then ANOVA was used, whether the groups differed and if the ANOVA-calculated p value was significant ($p \leq 0.05$; $p \leq 0.01$). Tukey's multiple-comparison *post hoc* test was performed to identify statistical differences between exposed groups and solvent control groups. There was no significant difference between the solvent and blank control, we therefore show only the results of the solvent control in the figures.

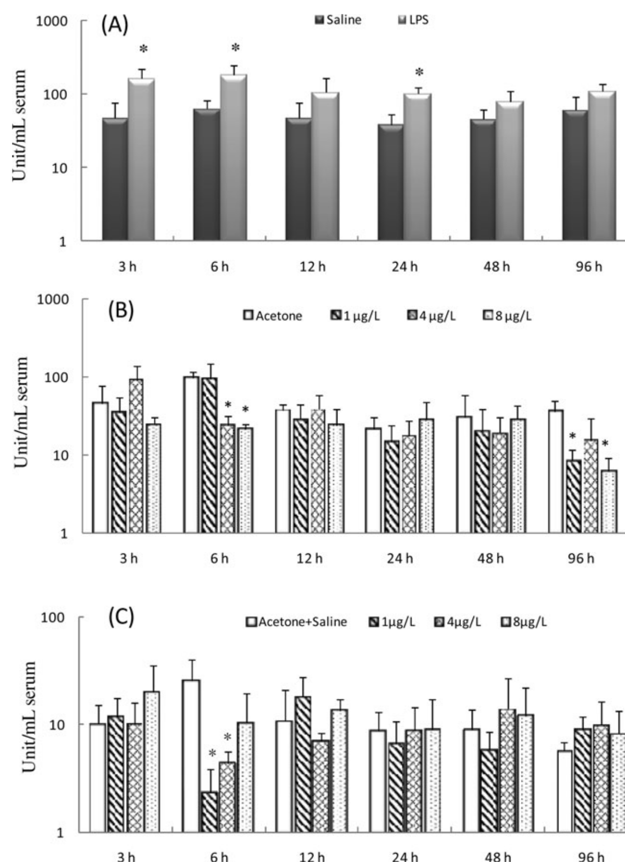

Fig. 1. Serum lysozyme activity of juvenile *P. major* challenged with 5 mg kg^{-1} BW LPS (A), exposed to different concentrations of BaP (B), or BaP waterborne exposure following challenge with LPS (C). An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

RESULTS

Serum Lysozyme Activity

The changes in the level of serum lysozyme activity are shown in Figure 1. The lysozyme activity increased in juvenile *P. major* after intraperitoneal (IP) injection of 5 mg kg^{-1} LPS from 3 to 96 h; however a significant increase was observed only at 3, 6, and 24-h post-LPS challenge compared to the respective control group [Fig. 1(A)]. When the fish were exposed to different concentrations of BaP there were no significant changes observed in lysozyme activity at 3, 12, 24, and 48-h postexposure, and the significant alteration of the serum lysozyme activity occurred at 6 and 96 h, respectively. The higher concentrations of BaP (4 and 8 μg L^{-1}) significantly reduced the lysozyme activity after 6 h exposure, however, after 96 h the lowest concentration (1 μg L^{-1}) and the highest concentration (8 μg L^{-1}) significantly reduced the lysozyme activity [Fig. 1(B)]. The effects of BaP exposure on lysozyme activity of juvenile *P. major* for 14 days followed by 5 mg kg^{-1} LPS challenge

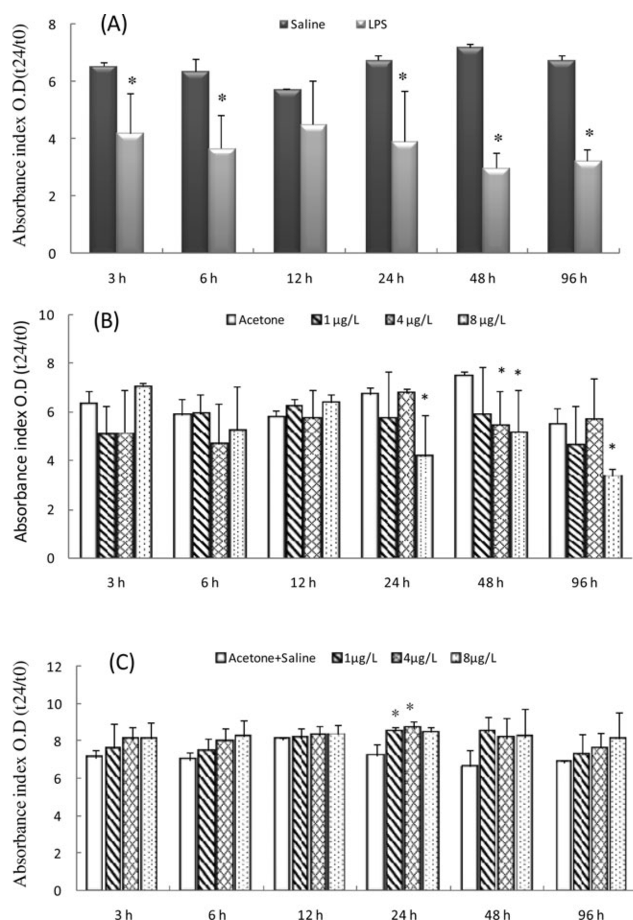

Fig. 2. Serum antibactericidal activity of juvenile *P. major* challenged with 5 mg kg^{-1} BW LPS (A), exposed to different concentrations of BaP (B), or BaP waterborne exposure following challenge with LPS (C). An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

are shown in Figure 1(C). The results revealed that there was no significant induction of lysozyme activity in comparison with the respective control group, except for 6 h after LPS challenge when the serum lysozyme activity significantly decreased in the fish exposed to 1 and 4 $\mu g\ L^{-1}$ of BaP.

Antibacterial Activity of Serum

The antibacterial activity of serum is shown in Figure 2. The absorbance index was markedly reduced in the serum collected from fish challenged with 5 mg kg^{-1} LPS from 3 to 96 h except for the 12-h time point [Fig. 2(A)], and this indicated that the bacterial growth was significantly inhibited after LPS challenge. When the fish were exposed to different concentrations of BaP there was no significant change of bacterial growth up to 12 h [Fig. 2(B)]. However, the antibacterial activity showed significant increase from 24 to 96 h after exposure to the highest dosage (8 $\mu g\ L^{-1}$). In addition, the bacterial growth was also inhibited after 48-h BaP exposure at a higher dosage (4 $\mu g\ L^{-1}$). The antibacterial activity in the serum of fish exposed to BaP followed by LPS injection did not show any significant change, but after 24 h the bacterial growth at BaP concentrations of 1 and 4 $\mu g\ L^{-1}$ was significantly greater than that of the respective control wells [Fig. 2(C)].

Respiratory Burst Activity

The respiratory burst activity of leukocytes significantly increased from 3 to 12 h compared to the saline-injected (LPS-free) control group after the fish were challenged with 5 mg kg^{-1} LPS. The activity showed its highest increase at 12 h, then decreased to the basal level from 24 to 96 h [Fig. 3(A)]. When the fish were merely exposed to different concentrations of BaP, the respiratory burst activity showed a significant increase from 6 to 12 h in all the BaP-exposed groups except for the 1 $\mu g\ L^{-1}$ at 6 h. No remarkable

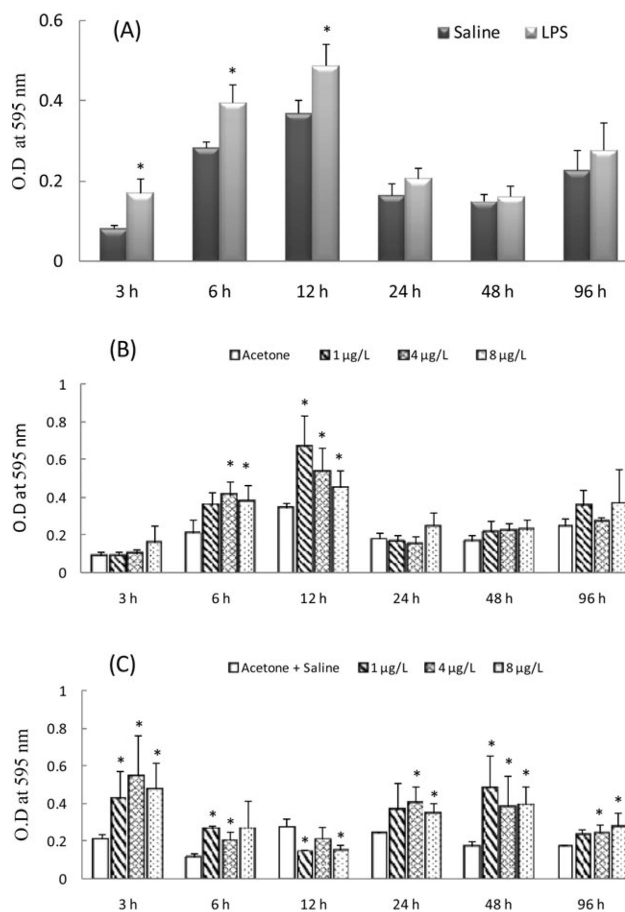

Fig. 3. The respiratory burst activity of leukocytes for juvenile *P. major* challenged with 5 mg kg^{-1} BW LPS (A), exposed to different concentrations of BaP (B), or BaP waterborne exposure following challenge with LPS (C). An asterisk (*) indicates statistical significance at $p < 0.05$ between the treatments and the corresponding controls.

response was observed from 24 to 96 h [Fig. 3(B)]. However, the respiratory burst activity was significantly enhanced in the fish that were exposed to BaP followed by LPS stimulation [Fig. 3(C)].

Quantitative Analysis of PM-hepc mRNA Expression

The PM-hepc mRNA expression patterns in the liver of juvenile *P. major* were evaluated using qPCR (Fig. 4). As shown in Figure 4(A), an upregulation of PM-hepc expression was observed at 3 and 6 h after LPS challenge and it was induced to ~13.4-fold and 6.1-fold, respectively in comparison with the calibrator. However PM-hepc mRNA expression decreased to the level of the control from 24 to 96 h.

When the fish were exposed to different concentrations of BaP (1, 4, and 8 $\mu g\ L^{-1}$) PM-hepc mRNA expression was altered during the whole exposure period from 3 to 96 h [Fig. 4(B)]. The significant increase of PM-hepc mRNA expression occurred at 96 h and the PM-hepc gene increased around 10.8-fold, 11.4-fold and 6.8-fold corresponding to the different concentration of BaP. In contrast, the mRNA expression of PM-hepc significantly decreased at 48 h when exposed to BaP at dosages of 1 and 8 $\mu g\ L^{-1}$, but no change was observed at the 4 $\mu g\ L^{-1}$ BaP concentration at that time. Interestingly, it was noted that the PM-hepc mRNA expression pattern was quite particular upon exposure to the highest BaP (8 $\mu g\ L^{-1}$) concentration [Fig. 4(B)]. Although some variation in PM-hepc mRNA expression occurred after 3 and 6 h, no significant change was observed. However, a significant decrease was present at 12 and 48 h, whereas a significant increase was observed at 24 and 96 h. In contrast, in fish exposed to a dosage of 4 $\mu g\ L^{-1}$ BaP, the PM-hepc gene maintained upregulation from 3 h to 96 h and it was significantly expressed at 12 and 96 h. However, no significant alteration of PM-hepc mRNA expression in the liver was observed when the juvenile *P. major* were exposed to different concentrations (1, 4, and 8 $\mu g\ L^{-1}$) of BaP followed by LPS challenge [Fig. 4(C)] at 3, 6, and 12 h. The PM-hepc mRNA expression was upregulated at 24 h at all three concentrations of BaP exposure. The expression level of the PM-hepc gene was significantly increased at 48 and 96 h and the level was ~2.6-fold, 4.1-fold, and 3.9-fold in comparison with the calibrator at 48-h post-LPS challenge, and ~2.9-fold, 7.6-fold, and 5.6-fold compared with the calibrator when the fish were exposed to different concentrations of BaP followed by 96-h LPS challenge [Fig. 4(C)].

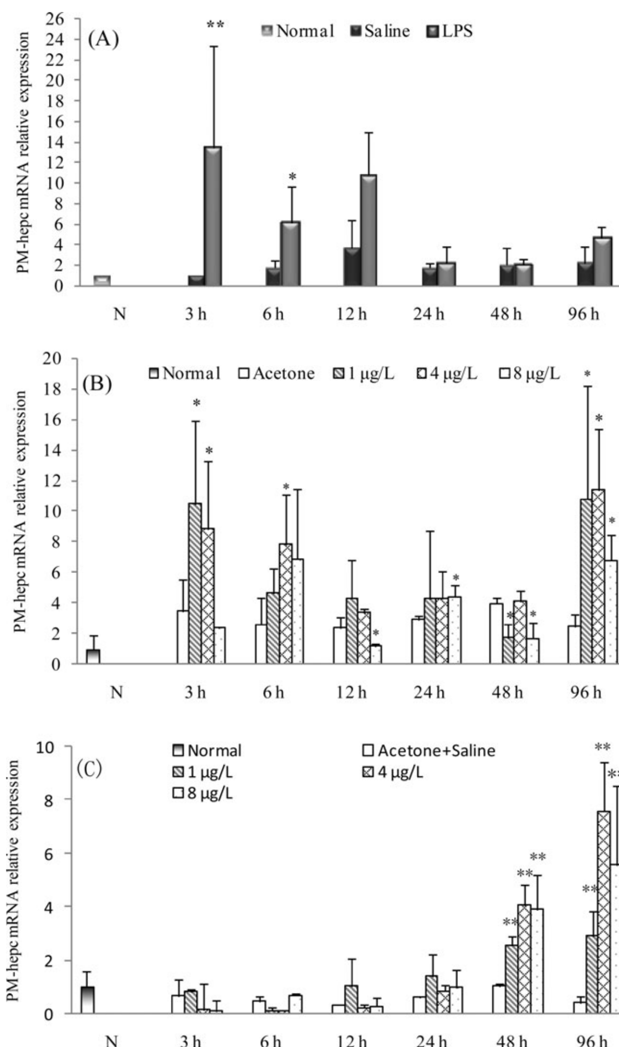

Fig. 4. PM-hepcidin mRNA expression with qPCR in liver of juvenile *P. major* challenged with 5 mg kg^{-1} BW LPS (A), exposed to different concentrations of BaP (B), or BaP waterborne exposure following challenge with LPS (C). Asterisks (*, **) indicate statistical significance at $p < 0.05$ or $p < 0.01$ between the treatments and the corresponding controls.

DISCUSSION

LPS is the major constituent of the external layer of gram-negative bacteria, and it activates host innate immunity by stimulating phagocytic cells to produce proinflammatory cytokines. It is considered to be a strong immunogen since both the ''O'' polysaccharide chain and the core region of the molecule can act as antigenic determinants (Jakobsen et al., 1999). LPS often serves as a protective antigen in many fishes against pathogens. As shown in the study, LPS challenge induced the activity of lysozyme and respiratory burst, antibactericial reaction, and the mRNA expression of PM-hepcidin gene, indicating these parameters are immunological responsive effectors. The positive response is generally thought as an increase of the antibacterial capacity present after the fish was challenged with LPS.

In general, BaP exposure alone had an inhibitory effect on the activity of lysozyme and serum bactericidal activity

but the effect was not much great. The results showed that the decrease of lysozyme activity was only present at 6 and 96 h with higher concentrations of BaP, and also the decrease of serum bactericidal activity was present from 24 to 96 h with the highest concentration of 8 μg L^{-1} BaP. The general trend of BaP effect on the lysozyme activity and serum bactericidal activity stimulated by LPS in fish was totally inhibitory. As observed in the study, the induction of lysozyme activity and serum bactericidal activity caused by LPS was diminished to the level of control when the fish was exposed to BaP for 14 days, indicating the significant inhibitory effect of BaP on both lysozyme activity and the serum bactericidal activity. Lysozyme is an enzyme that disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers. It acts directly on the walls of gram-positive bacteria, and on the inner peptidoglycan layers of gram-negative bacteria, after complement and other enzymes have disrupted the outer walls (Yano et al., 1996). This enzyme exists in most tissues and secretions of fish, and is most frequently examined in plasma or serum during toxicological studies (Bols et al., 2001), when its levels are regarded as a sensitive parameter (Reynaud and Deschaux, 2006; Sanchez and Porcher, 2009) and in fish studies as an indicator of exposure to various organic pollutants (Karrow et al., 1999). Lysozyme is also a major component of the fish innate immune system involved in inflammatory processes (Dautremepuits et al., 2009) and thus, the reduction of lysozyme activity observed in our study suggested that BaP had an immunotoxic effect upon the fish. Similar observations on the decrease in lysozyme activity in fish due to PAH exposure are reported (Tahir and Secombes, 1995; Secombes et al., 1997). The decreased lysozyme activities of *P. major* might make fish at a risk in bacterial infection. Similarly, the significant antibacterial activity in the serum of LPS-challenged fish disappeared when fish was exposed to BaP for 14 days and even the bacteria grew faster than the respective control at 24 h upon exposure to 1 and 4 μg L^{-1} BaP. The result indicated that the toxic effect of BaP significantly alleviated the serum antibacterial activity or inhibited the production of antibacterial activity of LPS challenged fish. Controversially, it was interesting to note that the highest concentration of BaP (8 μg L^{-1}) alone showed a significant inhibition of bacterial growth from 24 to 96 h, probably a resistance against bacteria occurred during the higher dosage BaP exposure. A similar observation was reported in a recent study in which rainbow trout (*Oncorhynchus mykiss*) were exposed to chlorothalonil following challenge with a pathogen (*L. anguillarum*) and the highest dosed group had the lowest mortality (Shelley et al., 2009). There is, however, limited knowledge regarding the possible defense generated from exposure to a pollutant, being a part of host immunity.

Contrary to the results of both lysozyme activity and the serum bactericidal activity, BaP exposure induced the respiratory burst activity at early 12 h and the mRNA expression of PM-hepcidin gene at more exposure time points. The general trend of BaP effect on the respiratory burst activity and the mRNA expression of PM-hepcidin gene was inducible. However, it is noteworthy that the induction pattern of respiratory burst activity and PM-hepcidin expression was significantly changed in comparison with LPS challenge alone when the fish was first exposed to BaP for 14 days and then challenged with LPS. The respiratory burst activity was significantly enhanced from 24 to 96 h, which is very different from that caused by either LPS challenge or exposure to BaP alone, showing a longer term of activity. Similarly, the PM-hepc gene was upregulated at 24 h and significantly increased at 48 and 96 h [Fig. 4(C)] while no significant expression was present at 3 and 6 h when the fish was challenged with LPS alone, indicating a delayed response to LPS challenge after fish was at first exposed to BaP for 14 days. It is known that respiratory burst (with the formation of superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals released by phagocytes) is an important mechanism for the protection of an animal against pathogens and for repairing its own damaged tissues (Thilagam et al., 2009); however, excessive superoxide anions can ultimately destroy some molecules at the mRNA level (Reynaud et al., 2008). In this study, the induction pattern of respiratory burst activity was changed when the fish was first exposed to BaP for 14 days and then challenged with LPS and its significant activity was extended until 96 h, which is much longer than that caused by either LPS challenge or exposure to BaP alone. The similar enhanced respiratory burst activity is also found in other study when fish are exposed to pollutants (Regala et al., 2001; Reynaud et al., 2001), and the increased production of superoxide anion is considered to be cytotoxic to the host (Cheng et al., 2007). Therefore, a high level of superoxide anion maintained in the fish as observed in our study may not be benefit but cause damages to the host animals, even though the respiratory burst activity could help the host eliminate the invading foreigners.

The hepcidin gene is identified in various vertebrates including fish (Shike et al., 2002; Ren et al., 2006; Wang et al., 2009b; Bo et al., 2011), and it responds to hypoxia, pathogen infection and iron overload (Rodrigues et al., 2006; Hu et al., 2007; Bo et al., 2011). Interestingly, some environmental contaminants such as TCDD, BaP, and PCBs (Volz et al., 2005; Falciani et al., 2008; Wang et al., 2009a) are reported to induce its mRNA expression, and the antimicrobial peptide defensin mRNA expression was also significantly induced of BaP-exposed *Mytilus edulis* (Brown et al., 2006). In this study, PM-hepc was significantly induced after BaP exposure [Fig. 4(B)], which is similar to the induction pattern previously observed in the study of As-hepc2 (Wang et al., 2009a). Although the mechanism underlining on BaP inducing hepcidin mRNA expression and the subsequent role is ambiguous some insightful information could be derived from the study. The

highly induced expression of hepcidin upon exposure to BaP may be a positive response related to an innate immune function as referred to one of many aspects of innate host defenses associated with acute inflammation (Hancock and Diamond, 2000), or may be involved in immune and/or inflammation-related pathways (Volz et al., 2005; Wrighting and Andrews, 2006). It is reported that the mammal cytokine interleukin-6 (IL-6) directly regulates hepcidin expression through the signal transducer and activator of transcription 3 (STAT-3) signal pathway (Wrighting and Andrews, 2006). Moreover, increased levels of IL-6 have been demonstrated in rats exposed to BaP (Stoian et al., 2007; Park et al., 2010), and BaP coordinately also can increase IL-6 expression in rat cell line (Umannova et al., 2011). It would be speculated that the accumulated concentration of BaP in fish will cause toxic effect as measured and in turn it would lead to an inflammatory response, which will induce hepcidin mRNA expression. However, this needs to be further investigation.

In this study, the PM-hepc mRNA expression pattern was altered and completely different from that with LPS challenge alone when the juvenile *P. major* were exposed to different concentrations of BaP for 14 days and then challenged with LPS. The delayed response to LPS challenge due to prior exposure to BaP may lead to a harmful effect on the innate immune defense of fish, which probably at least disturbs the regular pattern of immunity formed by the evolutionary process. In view of our results, it was observed that BaP exposure can either decrease the activity of lysozyme and serum bactericidal action or enhance the respiratory burst activity and PM-hepcidin expression, thus providing insightful information to us that the immunomodulatory mechanism of BaP effect on fish immunity will be complex and there might exhibit multiple ways in regulating functional genes or modulating the activity of immune associated parameters of fish upon exposure to BaP.

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