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Regulation of metallothionein gene expression in response to benzo[a]pyrene exposure and bacterial challenge in marine cultured black porgy (*Acanthopagrus schlegelii*)

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Abstract Metallothioneins (MTs) are normally considered to be sensitive indicators of heavy metal pollution, but it is less clear whether the MT gene can actively respond to other environmental stresses. In this study, an MT mRNA molecular sequence of 471 bp (full length) was identified in marine cultured black porgy (*Acanthopagrus schlegelii*), encoding 60 amino acids containing 20 cysteine residues. The MT sequence was highly homologous to that of other fish belonging to the MT superfamily type 1 family. The three dimensional structure of the deduced MT peptide was composed of two metal-binding domains capable of ligating divalent heavy metals. The MT mRNA transcripts were detected in the 11 tested tissues and the highest quantity was present in the liver. Stresses by two factors, benzo[a]pyrene (B[a]p) exposure and bacterial challenge, were evaluated on MT gene expression. The level of MT gene transcripts in the liver significantly declined 24 h post B[a]p exposure and the quantity was significantly correlated with the exposure time during a 24 h period. In contrast, MT gene expression in the liver was significantly increased 48 h post bacterial infection and the quantity was significantly correlated with the infection time during this period of 48 h. Our results indicated that MT gene expression in black porgy liver was sensitive to environmental stresses other than just the heavy metal pollution reported, suggesting that the development of a reliable biomarker for heavy metal pollution will be more complex than expected.

Key words metallothionein; environmental stress; *Acanthopagrus schlegelii*; gene expression

1 Introduction

Metallothioneins (MTs) are low molecular weight (6–7 kDa) proteins rich in cysteine residues which have been identified in many organisms since the initial discovery in equine kidney in 1957 (Margoshes and Vallee, 1957). Metallothioneins from both mammals and fishes have a conserved high density of 20 cysteine residues per protein molecule, and were determined experimentally or predicted to be made up of two metal-binding domains (Braun et al., 1992; Capasso et al., 2003; Klaassen et al., 2009). The primary physiological role of MT has been defined, after extensive studies, as the maintenance of homeostasis of essential metal elements and it is believed to be a sensitive indicator of heavy metal pollution in aquatic organisms (Amiard et al., 2006; Paul-Pont et al., 2010; Ladhar-Chaabouni et al., 2012). However, MT synthesis is regulated by a wide variety of other agents in vertebrate models, such as glucocorticoid hormones, cytotoxic agents, UV radiation, and inducers of oxidative stress; and the cytokines and immune stimulants are also effective inducers of mammalian MT (Jenny et al., 2006). Being a thiol containing protein, MT is a potentially effective free radical scavenger, and therefore, as a member of the non-enzymatic systems in

antioxidant defense, it is important in regulating the cellular redox-state and critical in protection against exogenous oxidants (Kling and Olsson, 2005).

Black porgy (*Acanthopagrus schlegelii*) is one of the most important commercially valuable marine fish in southern China. Usually raised in coastal water at high stocking density, black porgy has been presumed to be more tolerant than other fish species among the Sparidae family members, in confronting a variety of potential exogenous contaminants, including persistent organic pollutants such as benzo[a]pyrene (B[a]p), microbial organisms and traces of heavy metals. These exogenous toxicants can play a role as an oxidative stress, involving various physiological and pathophysiological, endogenous and exogenous processes that eventually directly or indirectly affect the oxidant/antioxidant balance in the cell (Ma, 2010). Metallothionein has been used as a biomarker to indicate the pollution caused by heavy metals in aquatic organisms, but study of the role of MT in other bio-processes is still limited. In this study, an MT molecular sequence in black porgy was isolated and characterized in terms of the three dimensional structure of the deduced peptide. The MT gene profiles in different tissues and their response to B[a]p exposure and bacterial injection were measured. Our aim was to assess the MT response to ubiquitous pollution by B[a]p and infection by bacteria in black porgy marine fish farms, and our results should shed light on the role which MT plays in enabling cultured fish to protect themselves and to survive in a poor living environment.

2 Material and methods

2.1 Fish rearing and experimental design

Juvenile black porgy (male, average weight 153 ±35.0 g and average length 17.8±1.39 cm) were obtained from the Tongan marine-culture farm in Xiamen, Fujian Province, China. Fish were acclimatized to laboratory conditions in seawater tanks for 7 d, and fed with commercial fish food pellets daily. Healthy fish were chosen and divided into three groups for the challenge experiment as follows: the blank control group, the bacterial injection group, and the B[a]p-exposed group. The acute bacterial infection experiment was separately performed for 48 h with no fish feeding. At 0 h, 50 μL of a live bacterial cocktail was intra-peritoneally injected into the fish. The cocktail was made up of four strains in sterile physiological saline solution at a final concentration of 10^{10} CFU/mL. These were *Staphylococcus aureus* CGMCC 1.363, *Escherichia coli* CGMCC 1.2389, *Vibrio parahaemeolyticus* CGMCC 1.1615 and *Micrococcus lysodeikticus* CGMCC 1.634, which were purchased from the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. For the water-borne B[a]p exposure experiment, B[a]p (HPLC grade; Sigma Chemicals, USA) was dissolved in acetone and then diluted in seawater to a nominal concentration of 1 μg/L. The exposure experiments were performed for 48 h, with the testing solution being renewed at 24 h intervals during this period and with no fish feeding.

2.2 Sample collection

Prior to the infection and exposure experiments, normal fish tissues were collected from the healthy fish (n=3) in the blank control group at 0 h. Blood cells were firstly sampled from the caudal vein using a syringe and then liver, spleen, head kidney, trunk kidney, heart, stomach, intestine, brain, gill and skin samples were collected from each individual fish, and immediately frozen in liquid nitrogen and then stored at -80℃. Liver samples from fish in the bacterial injection group and the B[a]p-exposed group were collected at 6 h, 24 h and 48 h post-challenge after fish blood had been collected, and livers were immediately frozen in liquid nitrogen and then stored at -80℃ for RNA extraction.

2.3 Cloning of MT complete cDNA sequence

Briefly, liver tissue from a healthy black porgy was ground quickly in liquid nitrogen using a mortar and pestle and transferred to 1 mL TRIzol Reagent (Invitrogen, USA), and total RNA was extracted according to the manufacturer's protocol. The 3' end of the MT cDNA was cloned by RACE method using a 3'- full RACE core set (TaKaRa). Firstly, cDNA was synthesized from 0.5 μg of total RNA using AMV reverse transcriptase XL and an oligo dT-3 site Adaptor Primer, and then 3' region of the MT cDNA was amplified with a designed degenerate primer F1 [5'CCT GAA AAA TGG AC(T)C CTT GCG AG(T)T G] and a 3 sites adaptor primer from the kit. The PCR cycles were set as follows: 94℃ for 3 min, followed by 30 cycles of 94℃ for 30 s, 60℃ for 20 s and 55℃ for 20 s, and 72℃ for 45 s; and extension at 72℃ for 10 min. The 5' end of the MT cDNA was cloned by RACE method using a SMART^{TM} RACE cDNA amplification kit (Clontech) following the manufacturer's protocol. Firstly, cDNA was synthesized from 0.5 μg of total RNA using PowerScript reverse transcriptase, SMART II A oligo and 5'-CDS primer. Then, cDNA products were diluted in a Tricine-EDTA buffer and 5 μL of this dilution was amplified as a template by PCR assay using a universal primer mixture from the kit and a designed gene-specific primer R1 (5'ACA CGC AGC CAG AGG CGC ACT TG 3'). The PCR

cycles were set as follows: 95℃ for 3 min; 5 cycles of 30 s at 95℃, 60 s at 72℃; 5 cycles of 30 s at 95℃, 30 s at 70℃, 30 s at 72℃; 25 cycles of 30 s at 95℃, 30 s at 68℃, 30 s at 72℃; and extension at 72℃ for 10 min. PCR products were analyzed by electrophoresis and the target DNA bands were purified using a QIAquick gel extraction kit (Qiagen), and then DNA fragments were ligated into pMD18-T vector (TaKaRa) and sequenced (Invitrogen, China).

2.4 Reverse-transcription, quantitative polymerase chain reaction (RT-qPCR)

Each tissue sample of liver, spleen, head kidney, trunk kidney, heart, stomach, intestine, brain, gill and skin was individually ground in liquid nitrogen and then total RNA was extracted using TRIzol Reagent. Blood cells from 200 μL of fresh whole blood were used for RNA extraction with 1 mL TRIzol Reagent. Aliquots of 0.5 μg of RNA were prepared to perform the reverse transcription reactions using a PrimeScript^{TM} reverse transcription Reagent Kit (TaKaRa) for Real-time PCR analysis. The MT gene and 18S rRNA gene in each tissue tested were amplified in duplicate in separate reactions using Power SYBR Green PCR Master Mix (Applied Biosystems, UK) with a 7500 Real Time PCR System (AB Applied Biosystem). MT gene expression was relatively quantified using 18S rRNA gene as an endogenous control gene. The primers used in real-time PCR were shown in Table 1. To obtain equal amplification efficiencies for two genes in expression quantification, a serial dilution of liver cDNA (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600) was prepared, and three different concentrations (50 nM, 100 nM and 150 nM) of gene primer pairs (Tab. 1) were set to plot relative standard curves. The PCR processing procedure was set at 50℃ for 2 min, 95℃ for 10 min (initial polymerase activation), 40 cycles of 95℃ for 15 s (denaturizing) and 60℃ for 1 min (annealing, extending and fluorescence data collection), followed by a heat dissociation curve protocol from 60 to 95℃ to validate the specificity of PCR product. The CT value (threshold cycle number) was obtained from the kinetic curve of the real time PCR assay. The relative gene expression of each tissue was presented as the fold difference to the liver and calculated using the formula $2^{-\Delta\Delta CT}$. The expression patterns of the MT gene during the time course of B[a]p exposure and bacterial challenge were investigated in the liver using the 0 h liver as the calibrator sample.

2.5 Statistical analysis

The SPSS (version 11.0) was used for statistical analysis, the data having been log-transformed to normality. Differences of relative gene expressions among treatments were evaluated using one-way ANOVA followed by Fisher's least significant difference tests. Differences with $p<0.05$ were considered to be significant. Bivariate correlation analysis was conducted using Pearson coefficient analysis (two-tailed) between levels of MT gene expression and the challenge time course, and the R value and significance value were obtained.

3 Results and discussion

A full-length MT cDNA of 471 bp was obtained from black porgy, which consisted of a 73 bp 5' untranslated region, a 183 bp coding region, and a 215 bp 3' untranslated region including a poly A tail (GenBank accession No. EU126549). The deduced peptide sequence was 60 amino acids in length and had a high content of cysteine residues (20 of 60). The molecular weight of the deduced MT peptide was 5965.9 Da, the p*I* 8.05, and the hydrophobic residue ratio 40%. Phylogenetic analysis of our MT-like black porgy peptide and other known fish MT peptides showed that it had high similarity with these other fish MTs and belonged to the MT superfamily type 1 family (Fig. 1A). The 3D structure was modeled using the SWISS-MODEL tools (Arnold et al., 2006), and the structure of the MT of the Antarctic fish (*Notothenia coriiceps*, 1M0J and 1M0G), the first 3D structure of a fish MT determined experimentally, was used as a template. The peptide was presumed to be composed of two metal-binding domains: an N-terminal beta domain (2^{nd} to 29^{th} residue, Fig. 1B) presumed to have the ability to ligate three divalent metal ions to nine cysteines, and a C-terminal alpha domain (31^{st} to 60^{th} residue, Fig. 1C) presumed to coordinate with four divalent metal ions via cysteinyl thiolate bridges from 11 cysteine ligands.

To obtain equal amplification efficiencies for the target gene MT and the endogenous gene 18S rRNA in real-time PCR within the range of experimental concentration, relative standard curves for each primer pair' concentration were plotted using log values of cDNA quantity as an *x*-axis and the corresponding CT values as a *y*-axis. The slope values of the relative standard curves using different primer concentrations were calculated and shown in Table 2. Two similar slope values were chosen (indicated by asterisk), between which the difference was less than 0.1, and as a result, the proper concentration of primers was decided as 50 nM for 18S rRNA and 100 nM for MT. Also, the melting curve analysis and the electrophoresis analysis of two amplicons of 18S rRNA and MT genes had been performed and indicated the specific and effective amplification of two genes.

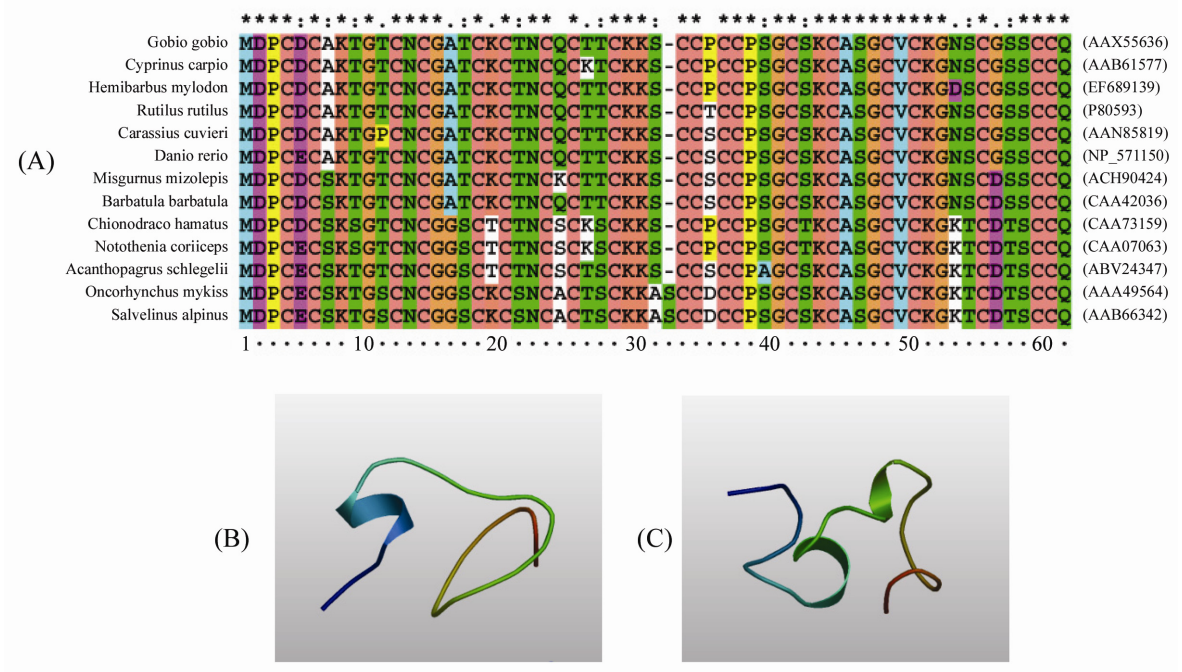

Fig. 1. The phylogenetic analysis (A) and the 3D stucture modeling of the deduced metallothionein (MT) peptide (B, C) from black porgy. (A) Clustal X alignment of amino acid sequence of MT from black porgy (underlined) with other known and predicted MTs. '*' Indicates positions which have a single, fully conserved residue; ':' indicates that one of the ''strong'' groups is fully conserved; '.' indicates that one of the ''weaker'' groups is fully conserved. SwissProt and GenBank accession numbers are shown in parentheses. (B) 3D structure model of the beta metal domain of black porgy MT (2^{nd} to 29^{th} residue). One β-sheet (indicated by the ribbon) by which the three divalent metal ions could be ligated. (C) 3D structure model of the alpha metal domain of black porgy MT (31^{st} to 60^{th} residue). Two β-sheets (indicated by the ribbon) by which the four metal ions could be ligated. The model was built with reference to the structure of metallothionein in the Antarctic fish (*Notothenia coriiceps*, 1M0J and 1M0G).

Table 1 The nucleic acid sequence of primers used in real-time PCR

Gene name	Primer (forward and reverse) 5' – 3'	Length of fragment	Accession number	Task
18S rRNA	Forward primer: ACAAAGGGCAGGGACTTAATCA Reverse primer: TCCCATGAACGAGGAATTCC	69 bp	AB259837	Endogenous control
Metallothionein	Forward primer: GCAACTGCGGAGGATCCTGCACATG Reverse primer: ACACGCAGCCAGAGGCGCACT TG	111 bp	EU126549	Target gene

The PCR amplicons of the MT gene were detected in all eleven tissues of black porgy tested, indicating that MT mRNA was ubiquitous in various tissues of the black porgy, which was consistent with the observations in other fishes (Cho et al., 2008). On comparing the quantity of MT mRNA among these tissues (Fig. 2), it was found that MT was most expressed in the liver and the quantity was nearly 1000 fold that in the gill, where MT was least expressed. In the brain, stomach and intestine, the mRNA quantity of MT gene was about 6 to 16 fold lower than that in the liver, but still 2 to 15 fold higher than that in the trunk kidney, blood cells, head kidney and spleen. While in the skin, heart and gill, the MT RNA was nearly negligible on comparing to the MT mRNA in the liver.

A down-regulated expression trend of the MT gene was observed in the liver during the time course of 48 h for B[a]p exposure (Fig. 3). A significant decline in MT mRNA quantity was found 24 h post B[a]p exposure compared with the control fish (p<0.05, one-way ANOVA). The results of the correlation analysis between the MT mRNA level and the exposure time within 24 h showed that R was equal to -0.72 at the 0.05 level, suggesting that a significant inhibition of MT gene expression was strongly correlated with B[a]p exposure within the 24 h time course.

An up-regulated expression trend of the MT gene was observed in the liver of black porgy during the 48 h time course after bacterial challenge (Fig. 4). A sig-

nificant increase of MT mRNA was found 48 h post bacterial challenge compared with the control fish (p<0.05, one-way ANOVA). Correlation analysis between the MT gene expression and the infection time within 48 h was conducted, and the results showed that R was equal to 0.719 at the 0.01 level, implying that a significant induction of MT gene expression was strongly correlated with bacterial infection within the 48 h time course.

Table 2 Slope values of the relative standard curves using different primer concentrations in real-time PCR

Gene name	Slope value			Fluorescence threshold
	50 nM	100 nM	150 nM	
18S rRNA	-3.27*	-3.11	-3.11	0.133
Metallothionein	-3.42	-3.20*	-3.39	0.188

Note: * The difference between two values is less than 0.1, so the primer concentration of 18S rRNA gene was chosen as 50 nM and that of MT gene was as 100 nM for quantitative real-time PCR analysis.

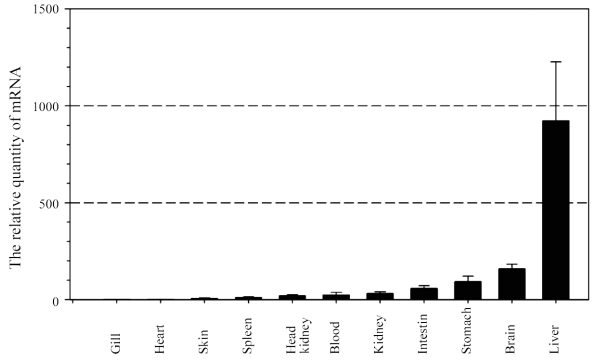

Fig. 2. Tissue distribution of metallothionein gene in healthy black porgy. The quantity of MT mRNA was normalized using 18S rRNA, and the gill was used as the calibrator (1 fold). Data are means ± S.E.M. (n=3, in duplicate).

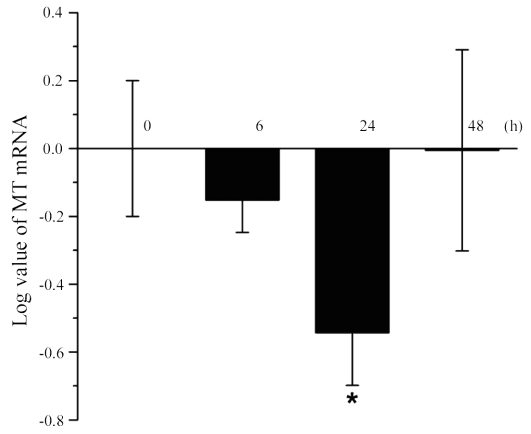

Fig. 3. The expression profiles of the metallothionein gene in the black porgy liver after B[a]p exposure. Data are means±S.E.M (n=3, in duplicate). '*' indicates statistical significance (p<0.05, one-way ANOVA).

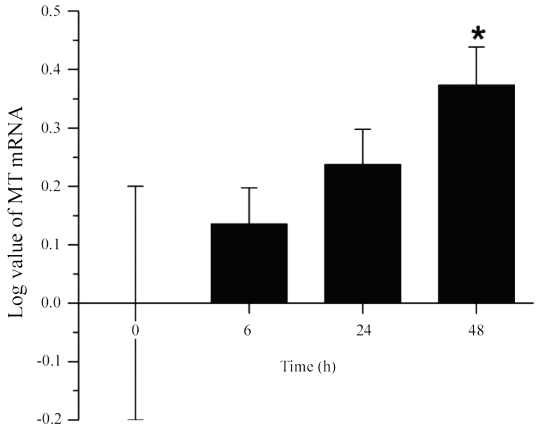

Fig. 4. The expression profiles of the metallothionein gene in the black porgy liver after bacterial injection. Data are means±S.E.M (n=3, in duplicate). '*'indicates statistical significance (p<0.05, one-way ANOVA).

Metallothioneins are considered to be metal binding peptides, and MTs from mammals and fishes were determined experimentally or predicted to be made up of two metal-binding domains, i.e., the N-terminal β domain and the C-terminal α domain, but the last CXCC motif of the α domain in the mammalian MT sequence becomes CXXXCC in the fish MTs, which consequently leads to a structural change of the α domain and, in turn, to a different charge distribution with respect to that observed in mammal MTs (Capasso et al., 2003). The MT peptide deduced from black porgy was analyzed as homogenic with other fish MTs and predicted to have two metal domains, and thus presumed to have the capability to ligate divalent heavy metals.

B[a]p is an environmental mutagen and one of the polycyclic aromatic hydrocarbons (PAHs) widespread in aquatic environments, such as in the Taiwan Strait of China, where the black porgy fish farms are located (Tian et al., 2008; Li et al., 2010). Our results showed that the MT gene expression in black porgy were inhibited within the 24 h post challenge by 1 μg/L B[a]p exposure. This observation is consistent with the reduced synthesis pattern of MT in mummichog (*Fundulus heteroclitus*) observed on the first day after B[a]p injection (Van den Hurk et al., 2000). The mechanism of B[a]p carcinogenesis is related to oxidative DNA damage exposed to reactive oxygen species (ROS), which might be the product of cytochrome family reaction such as the 4501A1 (CYP1A1) enzyme reaction in the metabolization and activation of PAHs on the aryl hydrocarbon receptor signaling pathway (Kim and Lee, 1997; Burczynski et al., 1999; Tsuji et al., 2011). Wang et al. (2009) have reported that CYP1A1 gene expression in black porgy is induced at a concentration of 1 μg/L B[a]p expo-

sure, implying that at that concentration ROS can be induced by B[a]p exposure. The inhibited MT gene expression in black porgy after B[a]p exposure might be associated with the oxidative stress response to B[a]p and likely via the AhR signaling pathway.

The bacterial challenge used in this study induces an immune-associated reaction (Yang et al., 2011). Metallothionein gene transcripts in black porgy were induced by that bacterial infection, implying that the bacteria had become an inflammatory stress and induced the MT expression in order to capture hydroxyl radicals and protect the black porgy from the toxicity of ROS. But, since MT expression can be enhanced by inflammatory stimuli such as interleukin (IL)-1, IL-6, and interferon-γ, the role of MT is presumed to be directly as an anti-inflammatory mediator dependant on the pathophysiologic condition of the organisms (Creti et al., 2010). Our results showed that the MT gene in black porgy was at least involved in the immune-associated response.

The correlation between MT content and metal accumulation in tissues is reported in many biological systems (Inoue et al., 2009). Our results showed that the change of MT gene expression in black porgy was induced either with B[a]p exposure or bacterial challenge, indicating that MT gene expression in black porgy was sensitive to multiple environmental stresses rather than just heavy metal pollution in aquatic environments. This indicated to us that the development of a reliable biomarker for heavy metal pollution will be more complex than it was expected.

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