Acute Effects of Microcystins on the Transcription of Antioxidant Enzyme Genes in Crucian Carp *Carassius auratus*

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ABSTRACT: Recent evidences suggested that oxidative stress may play a significant role in the pathogenesis of MCs toxicity. In the present study, the acute effects of microcystins on the transcription of antioxidant enzyme genes were investigated in liver of crucian carp i.p.-injected with 50 μg MC-LReq per kg body weight (BW). We reported the cDNA sequences for four kinds of antioxidant enzyme (GSH-PX, CAT, Cu/Zn SOD, and GR) genes, and evaluated the oxidant stress induced by MCs through analyzing the transcription abundance of antioxidant enzyme genes using real-time PCR method. The time-dependent change of relative transcription abundance and expression of the antioxidant enzyme genes were determined at 1, 3, 12, 24, and 48 h. The transcription abundance varied among antioxidant enzymes, with GSH-PX and GR down-regulation, and CAT and SOD significantly upregulation. Based on these data, we tentatively concluded that the oxidant stress was induced by MCs, and caused the different response of the antioxidant enzyme genes. © 2008 Wiley Periodicals, Inc. Environ Toxicol 23: 145–152, 2008.

Keywords: microcystins; mRNA expression; antioxidant enzymes; crucian carp (*Carassius auratus*)

INTRODUCTION

Eutrophication in fresh waters often leads to excessive proliferation of cyanobacteria, also called water blooms. The cyanobacterial blooms lower the quality of drinking or recreational water and increase the risk of toxicity to animal and human health, and has become an important environmental problem in China (Xie, 2006). Some cyanobacteria can produce a variety of toxins, of which microcystins (MCs) are the most widespread distributed (Carmichael, 1994). MCs are small monocyclic peptides composed of several seven amino acids and possess the general structure (-D-Ala-L-X-erythro-b-methyl-D-isoAsp-L-Y-Adda-D-iso-Glu-*N*-methyldehydro-Ala), in which X and Y are two variable L-amino acids. Different combinations of two L-amino acids give rise to many MC variants, and so far more than 70 kinds of MC isoforms have been reported (Sivonen and Jones, 1999). For instance, if the two L-amino acids are leucine-arginine (LR), tyrosine-arginine (YR), and arginine-arginine (RR), it gives name to MC-LR, MC-YR, and MC-RR, respectively.

MCs are potent liver toxins and have been implicated in poisonings of animals and humans (Matsushima et al.,

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1990; Pouria et al., 1998; Yuan et al., 2006). The toxicological mechanisms of MCs are under intensive investigation (Vajkova et al., 1998; Zurawell et al., 2005; Chen et al., 2006; Zhang et al., 2006), and several possible mechanisms have been put forward: (1) MCs are preferentially taken up into hepatocytes by multispecific bile acid transporters (Runnegar and Falconer, 1982), where they specially bind to the serine/threonine of protein phophatases 1 and 2A and inhibit enzyme activities (Carmichael, 1992), resulting in the alteration of hepatocyte shape and function (Matsushima et al., 1990; Batista et al., 2004; Malbrouck et al., 2004; Jayaraj and Rao, 2006; Sicińska et al., 2006); (2) At lower doses, MCs exposure cause the intestinal and liver dysfunction, as well as promotion of liver tumors and at higher doses, cause liver hemorrhage, necrosis, and hypovolaemic shock (Carmichael, 1994; Chen et al., 2004; Shi et al., 2004; Zurawell et al., 2005; Gonçalves et al., 2006); (3) MCs were proved to have genotoxicity, causing the damage of chromosome and DNA mutation (Rao and Bhattacharya, 1996; Zhan et al., 2004; Lankoff et al., 2006; Zegura et al., 2006).

Recently, more and more evidences have shown that oxidative stress may play a significant role in the pathogenesis of MCs toxicity (Ding et al., 1998; Li et al., 2003, 2005; Jos et al., 2005). The metabolism of MCs frequently results in the formation of reactive oxygen species (ROS), such as superoxide radical (O^{2-}) and hydrogen peroxide, which oxidize unsaturated lipids in cell membranes, proteins, and DNA, significantly contributing to their toxicity (Rao and Bhattacharya, 1996; Zegura et al., 2004; Sicińska et al., 2006). During the long evolution, two kinds of defense lines were developed to protect organisms from the damage of oxidants. The first defense line is the antioxidant molecules, such as Vitamin C and E, Uric acid, Glutathione (GSH), Selenium, Carotenoids, etc. (Gehringer et al., 2003; Alvarez et al., 2005). Another one is the antioxidant enzymes system, which was believed to play an important role in antioxidant defense in vertebrates. The ROS generated in tissues could be effectively scavenged by the antioxidant and GSH-related enzymes, such as glutathione peroxidase (EC 1.11.1.19, GSH-Px), superoxide dismutase (EC 1.15.1.1, SOD), and catalase (EC1.11.1.6, CAT), glutathione reductase (EC 1.6.4.2, GR), glutathione transferase (EC 2.5.1.18, GST) etc., preventing the cells from adverse effects of oxidative stress.

Aquatic organisms can be exposed to MCs via the consumption of toxic cyanobacteria, therefore, they are very good models to study the MCs toxicology and good biomarkers for the evaluation of MCs contamination in natural water habitats (Xie, 2006). To evaluate the oxidant stress induced by MCs, the response of antioxidant enzymes have been increasingly reported in fish species (Wiegand et al., 1999; Best et al., 2002; Li et al., 2003, 2005; Malbrouck et al., 2003; Jos et al., 2005; Cazenave et al., 2006; Prieto et al., 2006). However, most of the studies have been performed from a biochemical point of view, and less is known about the effects of MCs on antioxidant enzyme genes at transcriptional level (Wang et al., 2006). Therefore, it is necessary to detect the acute response of antioxidant enzymes on transcriptional level.

The main objectives of this research are (1) to report the cDNA sequences and the real-time PCR primers for antioxidant enzyme (GSH-PX, CAT, Cu/Zn SOD, and GR) genes in crucian carp; (2) to evaluate the oxidant stress induced by MCs through analyzing the transcription abundance of antioxidant enzyme genes using real-time PCR method, and this will provide new insights into the toxicological mechanism of MCs. Although accumulation of MCs in kidney, gonad, intestine, and gill were reported, fish liver is believed to be the mainly targeted organ (Chen et al., 2006; Xie, 2006), so liver tissue of crucian carp was used for this study.

MATERIALS AND METHODS

Chemicals

The cyanobacterial materials were from Lake Dianchi, Yunnan Province, China. The toxin content was determined via reverse-phase high performance lipid chromatography according to the method of Fastner et al. (1998). The MCs content was 1.41-mg/g dry weight (DW), with MC-LR, -RR, and -YR being 0.50-, 0.84-, and 0.07-mg/g DW, respectively. Crude algae were extracted with methanol, and finally suspended in 0.75% saline (0.75% sodium chloride injection).

Animals

Crucian carp *Carassius auratus* is a native species distributed extensively in freshwaters of China. About 500 adult specimens (about 150 g) were purchased from a fish farm near Huazhong Agricultural University, and transported to a big water tank (2 m $\times$ 2 m $\times$ 1.5 m) for 1 week. Fish were acclimated to laboratory conditions for 4 weeks prior to experiments. They were maintained in 150-L glass tanks (95 cm $\times$ 55 cm $\times$ 40 cm) containing dechlorinated tap water (pH = 7.6) under continuous aeration in temperature-controlled room with 12L:12D photoperiod. Water temperature was controlled at (25 $\pm$ 1)°C, and dissolved oxygen was (6.8 $\pm$ 0.7) mg L^{-1}. Feeding of pellet food at a rate of 1% of the body weight per day was terminated 2 days before initiation of the experiment, and no food was supplied to fish during the experimental period.

Experimental Protocol

A total of 120 acclimated crucian carp were injected i.p under ventral fin into the peritoneum by syringe. The doses

of ~1.0-mL suspension of extracted solution of MCs in 0.75% saline water, amounting to 50 μg MC-LReq per kg body weight (BW). Fish ($n = 60$) injected i.p. with 0.75% saline served as control. The 60 fish in the dose and the control group were respectively divided equally into 12 aquariums. Six fish samples from the dose group and the control were sacrificed at 1-, 3-, 12-, 24-, and 48-h postexposure to MCs. Liver tissues from control and dose group were separated, and frozen in liquid nitrogen until RNA extraction.

cDNA Cloning of Antioxidant Enzyme Genes

Total RNA was isolated from fish liver using TRizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of total RNA were determined by OD260/280 using an Eppendorff spectrophotometer (Eppendorff, Germany). The RT-PCR method was used to amplify the cDNA sequences of antioxidant enzyme genes using a Reverse ver 3.0 kit (Takara, Dalian, China). Reverse transcription was performed at 42°C for 40 min using oligo dT primers (10 μM) for Cu/Zn SOD, CAT, GSH-PXs, and GR, as well as a housekeeping gene GADPH in 50-μL total volume. The RNA concentration was 500 ng in each reaction.

Sets of degenerate primers for each antioxidant enzyme gene and the house keeping GAPDH gene were designed based on the homologous sequences from the GenBank (primers available on request but not shown here). PCR was performed to amplify expected cDNA sequences of antioxidant enzyme genes. Approximately 500 ng of cDNA were used as a template for 25-μL reactions containing 0.1 mM each dNTP, 10 mM each primer, 2.5 mL PCR buffer, and 0.5 units of Taq Ex polymerase (Takara, Dalian, China). PCR reactions were on a PTC-200 cycler (MJ research, USA) initially for a 94°C denaturing for 2 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 8 min. The PCR products were visualized by 1% agarose gel electrophoresis and ethidium bromide staining. The well-amplified bands were cut and purified using a gel recovery kit (Takara, Dalian, China) according to the manufacturer's instructions. The purified DNA was cloned and sequenced by Sangon Company (Shanghai, China). The cDNA sequences were searched for homologous sequences in GenBank database to check the specificity of the degenerate primers.

Real-time RT-PCR Analysis

Antioxidant gene mRNA expression was determined by real time RT-PCR using the house keeping gene GAPDH as reference. Total RNA was isolated from fish liver using TRizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of total RNA were determined by OD260/280 using an Eppendorff spectrophotometer (Eppendorff, Germany). The SYBR Premix Ex Taq (Takara, Dalian, China) was used for the real-time PCR and the protocol was according to the manufacture's instructions. The Real-time quantification analysis was on a Chromo4 Real-Time Detection System (MJ Research, USA). Briefly, PCRs were performed in a 20-μL volume containing SYBR Green 1 Mix (2 × concentration, Takara, Dalian) 10 μL, 200 nM forward primer, 200 nM reverse primer, and cDNA equivalent to that generated from 20 ng total RNA. Reactions were based on a two-step method initially for a 94°C denaturing for 10 s; 45 cycles at 94°C for 10 s, 60°C for 20 s.

We used the total RNA to make the standard curves for each gene according to the instructions of the SYBR Premix Ex Taq kit (Takara, Dalian, China). Standard curves were generated for each gene, and covered a range of cDNA equivalent to that generated from 1 to 200 ng RNA for all genes. For every antioxidant enzyme gene, quantitative PCR was performed in three triplicates and the mean normalized expression calculated from two independent MCs treated fishes. Normalized expression of each gene was calculated based on relative quantification method with respect to the expression of the house keeping gene GAPDH. The relative quantification was performed using standard calculations considering $2^{(-\Delta\Delta Ct)}$ in the Opticon Monitor software 2.03 Version (MJ research, USA), in which $-\Delta\Delta Ct = -[(Ct_{Target} - Ct_{GAPDH})_{Time\ x} - (Ct_{Target} - Ct_{GAPDH})_{Time\ 0}]$. Antioxidant enzyme expression levels were normalized to the GAPDH, and mRNA levels at 0 h were referred to as 1. For instance, at some time point, if $2^{(-\Delta\Delta Ct)} > 1$, it is upregulation; < 1, downregulation; $= 1$, unchanged.

Specific primers for real-time RT-PCR were designed based on the above-mentioned cDNA core sequences, and were synthesized by Sangon Company (Shanghai, China). The detailed primer sequences were given in Table I. Before the real-time PCR analysis, the specific primers were used for preamplification, and the PCR products were sequenced to check the specificity of the primers.

Statistics

The data were compared by repeated measures analysis of variance (ANOVA) using STATISTICA software package (Version 6.0, Statsoft). The data from dose and control fish are presented as means ± S.E.M., and $P \leq 0.05$ was considered statistically significant.

RESULTS

The Gene Cloning and the Real-time PCR Analysis

Degenerate primers were used for the cDNA amplification of each antioxidant enzyme gene, and the expected PCR

TABLE I. Nucleotide sequences of real-time PCR primers used for antioxidant enzymes

Accession Number	Gene Name	Forward/Reverse Primer	Size (bp)
EF116921	GSH-PX4a	CGCCAAGTATTCTGAGAGAGGTT GTTGTTCCTTCAGCCACTTCCA	191
EF139090	GSH-PX4b	CCAAAGGCTACAAGGCAGAG CGCTTTCATCCATTTCCAGA	89
EF139089	GSH-PX1	GAGGCACAACAGTCAGGGATTA CGTTCACACCGTTCACTTCCA	219
EF139091	SOD	GGTCCGCACTACAACCCTCA GCTATCACATTACCAAGGTCTCC	131
EF139092	GR	CATCCATCATCATACGGCAAG CCCAGAGCAGACAGTCCACCT	234
EF139093	CAT	CTGGAGTTTGCGTCCTGAATCGTT GTCCCTGAGCGTTGACCAGTTTGA	128
On submitting	GAPDH	GTCCGTCTTGAGAAACCTGCCA GGTCCTCAGTGTATCCCAGAATGC	300

products were purified and cloned for sequencing. After searching for the homologous sequences in GenBank database, it was confirmed that the core cDNA fragments of six genes (GR, Cu/Zn SOD, CAT, three GSH-PXs (GSH-PX1, GSH-PX4a, and GSH-PX4b) were successfully amplified. Using the Reverse ver 3.0 kit (Takara, Dalian, China), we got the all-long cDNA sequence for GSH-PX4a. All sequences have been deposited in GenBank (Accession nos. EF116921, EF139089, EF139090, EF139091, EF139092, and EF139093).

Based on the nucleotide sequences of the antioxidant enzyme cDNAs, six pairs of real-time PCR primers were synthesized. Before the real-time PCR analysis, the specific primers were used for preamplification, and the PCR products were sequenced and each pair of primers could satisfactorily amplify the corresponding antioxidant enzyme cDNA.

Standard curves were generated for each gene, and the target antioxidant genes and the selected reference GAPDH gene had very similar amplification efficiency (data not shown here). Therefore, the primers were proved very suitable for the real-time RT-PCR analysis of gene expression in crucian carp.

Response of Antioxidant Enzyme Genes Postinjection

The relative transcriptional abundance of antioxidant enzyme genes using the delta delta Ct method was shown in Figure 1. The time-dependent changes of gene transcription level were observed for the six enzyme genes in crucian carp liver after injection of 50-μg MCs/kg body weight dose. As the whole, the change of transcription abundance varied among antioxidant enzymes, with GSH-PXs and GR downregulation, and CAT and SOD upregulation significantly.

The time-dependent changes of transcription abundance for antioxidant enzyme genes were observed. Compared with the control, the transcript level of GSH-PX1 gene significantly decreased at all the time points (0.15-fold), with slight elevation at 3 h. The transcript level of GSH-PX4b decreased all the time points with slight elevation at 24 h. For the GR, the transcription abundance significantyl down-regulated at 3, 12, and 24 h (0.6-fold) except for slight increase at 1 and 48 h. The transcription abundance of Cu/Zn SOD increased significantly all the time points but the time-dependent change was not obvious. The transcription abundance for CAT significantly ($P < 0.05$) upregulated all the time points with a sharp decrease at 12 and 24 h.

DISCUSSION

Although the responses of antioxidant enzymes induced by MCs have been increasingly investigated, the reports on fish species were very limited and mainly focused on the changes of enzyme activities. The increases of antioxidant enzyme activities are typical observations in fish species exposure to MCs (Wiegand et al., 1999; Li et al., 2003; Jos et al., 2005; Cazenave et al., 2006). For instance, Wiegand et al. (1999) observed the increased enzyme activities in zebrafish embryos exposure to 0.5 μg/L MC-LR. The activities of SOD, CAT, and GSH-PX increased significantly after 6-h exposure in hepatocytes of common carp exposed to 10 μg/L MC-LR, in loach after oral exposure to MCs (Li et al., 2003, 2005), and in tilapia fed with cyanobacterial cells (Jos et al., 2005). Cazenave et al. (2006) reported that the activities of GR, GSH-PX, and CAT were enhanced in

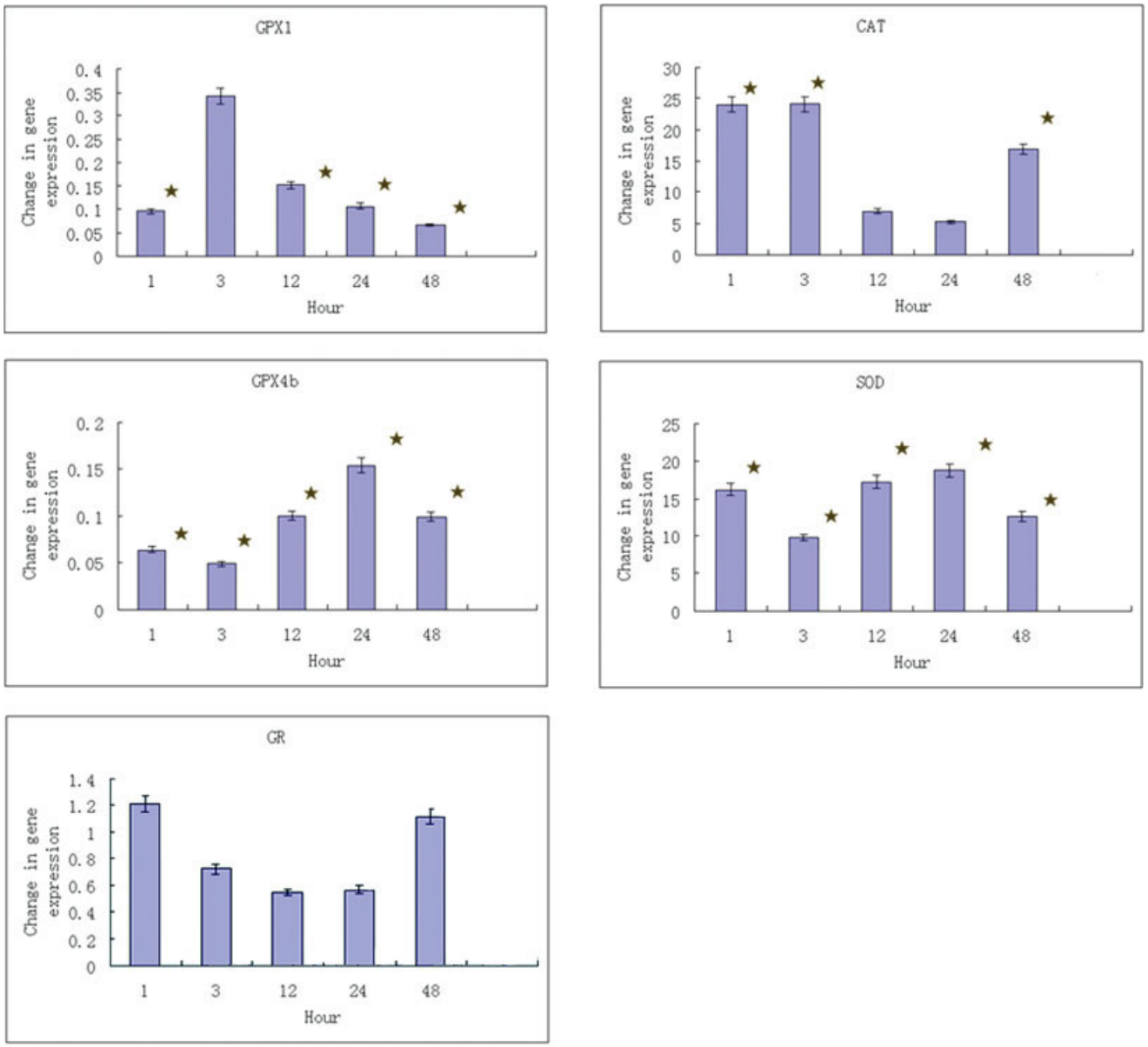

Fig. 1. The transcriptional abundance dynamics of antioxidant enzyme genes in 48 h in liver of crucian carp i.p. injected with 50 μg MC-LReq per kg body weight using the delta delta Ct method. Data are given as means $\pm$ S.E.M. error. Asterisk (*) indicates significance ($P < 0.05$) compared with 0 h (no MCs exposure). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

liver of *Corydoras paleatus* exposed to 2 μg/L MC-RR. It is believed that the increases of SOD, CAT, and GSH-PX activities revealed that these antioxidant enzymes might play important roles in eliminating the excessive ROS. Recently, the effects of MCs on the transcriptional level of certain antioxidant or GSH-related enzymes have been reported. Wang et al. (2006) characterized MCs detoxification-related liver genes in Nile tilapia, and detected the gene expression using semiquantitative RT-PCR methods. Our group has detected the acute effects of MC-LR on the transcription of nine glutathione *S*-transferase genes in common carp (Fu and Xie, 2006).

In this study, the acute effects of MCs on the transcription of antioxidant enzyme genes in crucian carp were reported using real-time PCR method. The time-dependent changes of transcription abundance for antioxidant enzyme genes were observed in 48 h, and the gene expression varied among antioxidant enzymes, with GSH-PX1, GSH-PX4b, and GR downregulation, and CAT and SOD upregulation significantly. In the study, the oxidant stress index

(OSI), such as total glutathione (tGSH), oxidized glutathione (GSSG), Lipid peroxidation, and other important antioxidant molecules (Vitamin E etc) were not determined, so the evaluation of oxidant stress induced by MCs was limited. Nevertheless, we proposed the possible mechanisms that lead to the different response of antioxidant enzyme genes. After the injection of MCs in crucian carp, the ROS was generated, and the lower-molecular-weight antioxidant molecules, such as Vitamin E, Uric acid, Glutathione (GSH), and Carotenoids etc., respond rapidly for protection the cell from the oxidant damage. However, this first defense line could not effectively scavenge the excess ROS induced by 50 μg MCs/kg body weight (BW) dose. The gene expression level of SOD increased significantly, and more enzymes were synthesized to catalyze the highly reactive superoxide anion to H_2O_2. At the same time, the gene expression level of CAT increased to synthesize more enzymes to catalyze the increasing H_2O_2 to H_2O. Of the four kinds of enzymes, GSH-PX directly uses the GSH as substrate to catalyze the reduction of hydroxyperoxides to water at the expense of GSH. The decline of GSH concentration probably led to the decrease of transcript activities of GSH-PXs and GR (Ding et al., 2003; Moreno et al., 2005; Jayaraj et al., 2006). The slight decrease of GR expression after 1-h acute postinjection of MCs was observed, which indicated a decrease in the transfer of GSSG to GSH because of low levels in GSH and NADPH (Chance and Boveris, 1980). Based on these data, we tentatively concluded that the oxidant stress was induced by MCs, and caused the different response of the antioxidant enzyme genes.

Because the enzyme activities were not measured in this study, the correlation of the gene expression and the enzyme activities was not yet clear. According to other researcher's published data about enzyme activity in fish species (Li et al., 2003; Jos et al., 2005; Cazenave et al., 2006), the activities and gene expression of SOD and CAT were positive correlation, with GSH-PX and GR negative correlation. Considering the monitoring of pollution in natural waters, the suitability of SOD, CAT, and GSH-PX mRNA expression as biomarkers in fish may therefore be limited unless some clear relationships were established.

According to published papers, we found the gene expression dynamics of antioxidant enzymes exposure to MCs varied among fish species. The response of antioxidant enzymes in fish species was influenced by many biotic and abiotic factors, such as age, species selection, nutrition factors, feeding behavior, environmental factors, and so on (Rudneva, 1999; Alvarez et al., 2005). For instance, Wang et al. (2006) found that no significant changes were observed in the GSH-PX mRNA expression in the liver of tilapia exposed to MC-LR. In the liver of Nile tilapia (*Oreochromis niloticus* sp.) exposed to either MC-LR or MC-RR, enhanced CAT and SOD activities were observed, but no discernible effects were observed in GSH-Px activity (Prieto et al., 2006). Different activities were also observed in liver, gill, intestine of *Corydoras paleatus* exposed to MC-RR (Cazenave et al., 2006). The activity of GSH-PX raised in liver and intestine, while it was inhibited in gills. GR activity showed no change in intestine, but increased in liver, gills, and brain. Our previous studies suggested that the accumulation and distribution of MCs in aquatic animals were different in kidney, gonad, intestine, and gill (Chen et al., 2006), which suggested different oxidant stress in different organs, resulting in the different response of antioxidant enzymes in diversity organs.

We found that after exposure to MCs, the response of antioxidant enzymes in mice and rat was completely different compared with fish species. The enzyme activities of GSH-PX, GR, SOD, and CAT in the liver were decreased in MC-LR treated rats and mice (Moreno et al., 2005; Jayaraj et al., 2006), but significantly increased in fish species (Li et al., 2003; Jos et al., 2005; Cazenave et al., 2006). Fish are significantly less sensitive to MCs than mammals (Williams et al., 1995; Zurawell et al., 2005), which might partially interpret the different response of antioxidant enzymes. It has been reported that antioxidant enzymes can be correlated with phylogenetic position, with more ancestral biota exhibiting less activity (Alvarez et al., 2005). Recent researches showed that the activities of CAT, SOD, and GSH-PX in fish were lower than that of mammals and birds (Perez-Campo et al., 1993; Rocha-e-Silva et al., 2004). Another possible explanation for the differences between fish and mammals was that fish had to upregulate the antioxidant enzyme activities in order to have enough ability to prevent the damage from ROS exposure to MCs.

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