Acute Effects of Microcystins on the Transcription of 14 Glutathione *S*-Transferase Isoforms in Wistar Rat

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ABSTRACT: The glutathione *S*-transferases (GST) play important roles in the detoxification of microcystins (MCs). For better understanding of the responses of GST isforms to MCs exposure, informations about the effects of MCs on GSTs are necessary. In this experiment, we cloned the full length cDNA of 14 GST isoforms (GST alpha, kappa, mu, omega, pi, theta, zeta, and microsomal GST) from Wistar rat. The mRNA abundance of each rat GST isoform in the liver, kidney, and testis was analyzed by real time quantitative PCR. Multiple GST isoforms were constitutively expressed in all examined organs, but some isoforms were expressed at higher level in one organ than in others. The relative changes of the mRNA abundance in the liver, kidney, and testis of Wiatar rat i.v. injected with crude MCs extract at dose of $1LD_{50}$ were also analyzed. Generally, the expression of most GSTs in the liver and testis was suppressed while that in kidney was induced after being injected with MCs. It is suggested that the transcription of GST isoforms varied in different ways within an organ and between organs of Wistar rat exposed to MCs. © 2009 Wiley Periodicals, Inc. Environ Toxicol 26: 187–194, 2011.

Keywords: microcystin; glutathione *S*-transferases; real time PCR; gene expression; Wistar rat

INTRODUCTION

The occurrence of toxic cyanobacterial blooms in eutrophic lakes, reservoirs, and recreational waters is a worldwide problem (Paerl et al., 2001). Among cyanotoxins, microcystins (MCs) are considered to be one of the most dangerous groups and are known as a group of cyclic polypeptide hepatotoxins of varying potency (Chorus and Bartram, 1999). The production and release of MCs are related with cyanobacteria blooms. MCs, the secondary metabolites of toxic cyanobacteria, are a family of extremely toxic compounds produced by freshwater cyanobacteria belonging to the genera *Microcystis, Anabaena, Nostoc*, and *Oscillatoria* (Sivonen and Jones, 1999). So far, more than 80 structurally different MCs have been found, among which microcystin-LR (MC-LR) is the most common variant, followed by microcystin-RR (MC-RR) (Fastner et al., 2002). Recently, chronic exposures to natural MCs in drinking water and contaminated fisheries products are found to cause substantial damage to human health (Chen et al., 2009).

MCs are potent hepatotoxins due to their inhibition of several serine/threonine protein phosphatases including PP1 and PP2A, leading to the disruption of normal cell metabolism and functions. Acute MCs poisoning in animal is characterized by disruption of hepatic architecture, which is related to massive intrahepatic hemorrhage and death in a few hours. Chronic uptake of MCs results in generalized hepatocyte degeneration with necrosis, progressive fibrosis, deregulation of the apoptotic machinery, and mononuclear leukocyte infiltration (Mackintosh et al., 1990; Bhattacharya et al., 1997). Meanwhile, MCs have also been

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reported to accumulate in other organs such as kidney (Williams et al., 1995; Mohamed et al., 2003) and gonads (Chen and Xie, 2005; Ding et al., 2006). It is suggested that the formation of microcystin glutathione conjugate mediated by glutathione *S*-transferase (GST) is the first step in the detoxification of MCs in both mammals (Kondo et al., 1996) and a wide range of aquatic organisms (Pflugmacher et al., 1998; Zhang et al., 2009).

GST enzymes are found from bacteria to humans. They exhibit a broad and overlapping substrate specificity (Mannervik and Danielson, 1988), which makes it difficult to identify and characterize individual isoforms based solely on their catalytic properties. There are commonly 12 reported isoforms of glutathione transferase in mammals, including alpha, mu, pi, theta, sigma, omega, zeta, kappa, and microsomal GSTs (Hayes et al., 2005). Either induced or inhibited activity of GSTs after exposure to MCs was reported in different species. GST activity was elevated in the mice exposed to a single 75% LD_{50} dose of MCs (Gehringer et al., 2004), but inhibited in diverse organs of C*orydoras paleatus* exposed to microcystin (Cazenave et al., 2006). Different transcription of GST superfamily genes after exposure to MCs was also reported. Fu and Xie (2006) reported GSTs (and MGST) from liver of common carp, in which the transcription of GST alpha, theta, and pi changed greatly after being injected with MC-LR, while mu isoform was relatively stable, and Liang et al. (2007) reported that cyprinid fishes may show an alpha-class predominant expression pattern of liver GST genes. Li et al. (2008) and Hao et al. (2008) investigated the expression of different isoforms of GSTs in the various tissues and found that that transcription of GST isoforms varied in different ways within an organ and among organs of goldfish exposed to MCs. Knight et al. (2007) investigated the constitutive mRNA expression of 19 different GST enzymes in 14 different tissues in mice and found different expression of GSTs in various organs, suggesting different roles of many GST isoforms in detoxification of ingested xenobiotics. However, information of rat GSTs is still limited, and the role of the GST superfamily genes in different organs in the detoxification of MCs has never been studied in this species. The rat GST sequences have high similarity with those from human. It appears that the study on rat may shed some light on the potential mechanisms involved in detoxification of the algal toxin in human.

Based on these facts, a better understanding of GST isoenzymes is necessary to improve the knowledge of rat GSTs and mechanisms involved in toxicity of MCs. In the present experiment, we cloned 14 complementary DNA (cDNA) sequences of Wistar rat GSTs and examined the mRNA abundance of various GST isoforms in Wistar rat in a number of organs. We also compared the changes in transcriptional level of GST isoenzymes of Wistar rat after i.v. injected with cyanobacterial crude extract. The main purposes were to evaluate mRNA abundance and the time-dependent responses of various GST isoforms in diverse organs of Wistar rat exposed to MCs and to discuss the possible mechanisms underlying these observations.

MATERIALS AND METHODS

Animals

Male Wistar rats (8 weeks of age, weighing $200 \pm 20 \times g$) were obtained from the Center for Disease Control of Hubei Province, China. They were kept at 20°C with a 12-h light–dark cycle in stainless steel cages and given free access to standard rodent pellet diet and water. All procedures carried out on rats were approved by the Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

Toxin

Cyanobacteria (mainly composed of *M. aeruginosa*) were collected from surface bloom scum of Lake Dianchi, Yunnan Province, China. In this study, freeze-dried algae cells were extracted three times with 75% methanol (V/V). The extract was centrifuged, and the supernatant was applied to a C18 reversed phase cartridge, which had been preconditioned by washing with 100% methanol and distilled water. The cartridge was then washed with water and eluted with methanol. The elution was evaporated to dryness, and the residue was dissolved in distilled water for the toxic experiment. MCs content in the extracts was determined by high-performance liquid chromatography (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). Determination of cellular MC was carried out according to Li et al. (2005). The microcystin content was 87-μg MC-LR equiv/mL, among which MC-RR and -LR were 167.7 μg /mL and 47.0 μg/mL, respectively. Crude MCs extracts were finally suspended in salt solution water (0.9% NaCl).

LD_{50} of Cyanobacterial Crude Extracts Containing MCs

Male Wistar rats ($n = 40$) were divided into five groups, and rats in each group were administered by an i.v. injection of MCs at different dose of MC-LR equiv/kg body weight. Calculating the death number and mortality of rats in each group during 24 h, we obtained the LD_{50} level for 24 h by using the formula $LD_{50} = \log - 1[X_m - I(\Sigma p - 0.5)]$ (X_m, the log of max dose; p, mortality; Σp, the sum of mortality in each group; I, the difference between the log of adjacent two group dose). All animal procedures were approved by the Institutional Animal Care and Use

TABLE I. PCR conditions used in this paper

RT-PCR	RACE-PCR	Real-time PCR
94°C for 3 min	95°C for 3 min	94°C for 4 min
94°C for 30 s	95°C for 30 s	94°C for 20 s
(30 cycles)	(30 cycles)	(40 cycles)
52°C for 30 s	64°C for 30 s	62°C for 20 s
(30 cycles)	(30 cycles)	(40 cycles)
72°C for 1 min	72°C for 1 min	72°C for 25 s
(30 cycles)	(30 cycles)	(40 cycles)
72°C for 5 min	72°C for 5 min	72°C for 5 min

Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

Experimental Protocol

Healthy male Wistar rats weighing 200 ± 20 g were divided into two groups randomly. One group received intravenous injection of 1-mL microcysitn extracts at LD_{50} of 87 μg MC-LR equiv/kg body weight. As control, the other group was injected with the same volume of 0.9% saline solution. Rats in the two groups were sacrificed at 0, 1, 2, 4, 6, 12, and 24-h postinjection, respectively. Five rats were sacrificed at each time point. The liver, kidney, and testis samples were quickly removed, minced, and stored frozen in liquid nitrogen until RNA isolation.

Total RNA Extraction, Reverse Transcription, and Rapid Amplification of cDNA End

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified by determination at OD_{260}. The purified total RNA (2 μg) was then reverse transcribed. Reverse transcription was performed with oligo(dt)18 primer using first strand cDNA synthesis kit (TOYOBO, Japan). The mRNA sequences of human and mouse genome were searched database for homologous genes, and degenerate primers were designed from conserved regions to clone partial GST cDNA sequences of Wistar rat by Polymerase Chain Reaction (PCR). The PCR cycling conditions were listed in Table I. PCR products were cloned and sequenced, and based on the result of sequencing, gene-specific primers were designed for 5′-RACE and 3′-RACE.

Q-PCR to Determine the Levels of Gene Expression

All the primers used in the quantitative real-time PCR (Q-PCR) are listed in Table II. Because of >90% similarity, one pair of primers was designed to recognize both GSTa1 and GSTa2 isoforms. The specification of each pair of primers was confirmed by randomly sequencing six clones and further confirmed by the melting curve analysis using Q-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid, and only primers with similar amplification efficiency were used in this experiment. The plasmid concentration of each GST isoform was measured at OD_{260}, and the corresponding copy numbers were calculated based on the formula that 1 μg of 1000 bp DNA equivalent to 9.1×10^{11} molecules.

To examine organ distribution of GST isoforms in three organs, liver, kidney, and testis were dissected out from five rats without any treatment for total RNA isolation. Quantitative real-time RT-PCR was conducted by amplifying 1.0 μL of cDNA from the samples, with the SYBR Green qPCR kit (Finnzymes) on a Chromo4 Real-Time Detection System (MJ Research). Amplification conditions were listed in

TABLE II. Real time PCR primers used in this experiment

	Primer sequence (5′–3′)		
Target gene	Forward	Reverse	Size (bp)
Alpha 1/2	CCACCTGCTGGAACTTCTCCTCTAT	AGGCTGCTGATTCTGCTCTTGAAGG	97
Alpha 3	GCAGACCAGAGCCATTCTCAACTAC	ACGGTTCCTTGCTTTGTCCTTGATT	196
Alpha 4	TGCTACTGACACAGACCAGAGCCAT	TCTCTCCTTCAGGTCCTTCCCATAC	100
Kappa	TCCTGGCTGGGCTTTGAGGTCCTAT	CTGGTGGTTGGTTTCCACTGTCTTTCA	115
Mu 1	GGCGACGCTCCCGACTATGACAGAA	AATCCGCTCCTCCTCTGTCTCTCCA	180
Mu 2	CGCCATTCGCCTGTTCCTGGAGTAT	TCCACACGAATCCTCTCCTCCTCTG	249
Mu 3	CCTGCTCCTGGAATACACAGACTCG	GTCCACACGAATCCTCTCCTCTTCT	241
Omega 1	TTGAGAAGAATCCCTTTGGGCTGGT	AACCAGAGACGGCACCTTTGAGAAT	191
Omega 2	CTGGTTTGAGCGACTGGATGTAT	ATAGAGATTCAAGAAGCCCAGGAAA	151
Pi	TTGAGGCACCTGGGTCGCTCTTTAG	GGTTCTGGGACAGCAGGGTCTCAAA	205
Theta 1	ATGGCAGCATACGACCCTTCGGAGA	GAGATGTGCGGTCCGACAAGGAAGT	192
Theta 2	AAAGCACTGCCATCTTGATTTACCT	TACTATTTCTGTTCCGTTCCACCTT	219
Zeta	CAAGTGGGACAGGAGAACCAGATGC	GCCAGCAGTGCCTTGTTGATGTGAC	224
MGST	GCATTCCAGAGGCTAACCAACAAGG	CGATACCGAGAAAGGGAACGATGTT	166
GAPDH	GTCATCAACGGGAAACCCATCACCA	CTCCACGACATACTCAGCACCAGCA	90

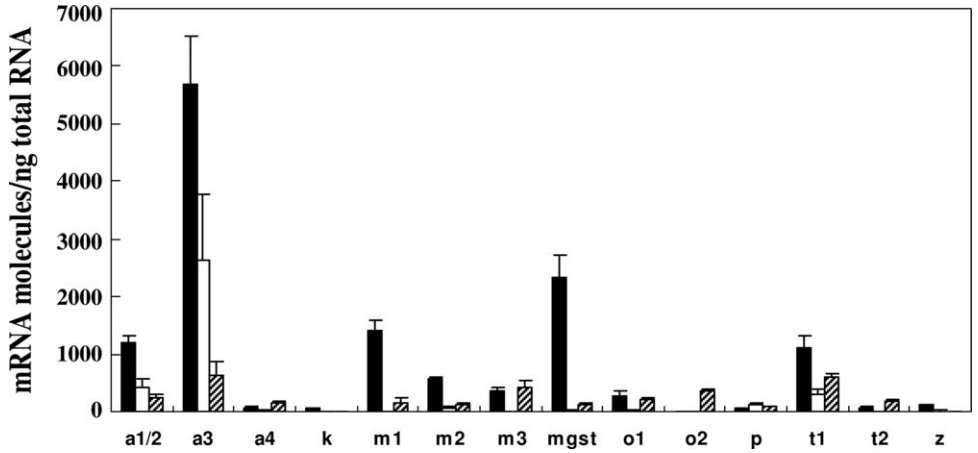

Fig. 1. The absolute abundance of transcripts of fourteen glutathione *S*-transferase isoforms in Wistar rat. ■ represents liver; □ represents kidney; ▨ represents testis.

Table I. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in three tubes, and PCR reactions without the addition of the template used as blanks. The absolute standard curve of each isoform was constructed in the range of 10^4–10^9 plasmid molecules. After completion of the PCR amplification, data were analyzed with the OpticonMonitor software 2.03 Version (MJ research), and the molecular copy of each GST isoform was derived from corresponding threshold value compared with standard curve.

To study the kinetic changes of rat GSTs transcripts temporal changes of rat GST transcripts after MCs stimulation, one commonly used housekeeping gene GAPDH was used as the internal control gene for the Q-PCR assay. Q-PCR was conducted as mentioned earlier. The δC_t method was used for quantification of amplified gene targets according to the manufacturer's protocol (Applied Biosystems). The relative expression levels (fold induction) of the genes were presented as $2(-\Delta\Delta C_T)$ method (Schmittgen and Zakrajsek, 2000; Livak and Schmittgen, 2001).

Statistics

All values are expressed as mean ± standard deviation. One-way ANOVA was used to elucidate if there are significant differences between the treatment groups and the control group ($P < 0.05$).

RESULTS

Molecular Cloning of GST Sequences

The full open-reading frames of GST isoforms of GSTa1/2, GSTa3, GSTa4, GSTk, GSTm1, GSTm2, GSTm3, MGST, GSTo1, GSTo2, GSTt1, GSTt2, and GSTz were cloned (The Genbank accession numbers of these sequences were FJ179397-FJ179410.)

Transcription of Various GST Isoforms in Different Organs

The typical standard curve for GST isoforms and GAPDH were constructed using a series of 10-fold dilutions of each plasmid DNA molecules from 10^4 to 10^9. For each assay, the linear correlation ($r^2 > 0.99$) between the crossing point and log concentration of GST isoforms or GAPDH mRNA copy number was observed. The amplification efficiencies during the exponential phase were highly reproducible.

All the GST isoforms studied in the present experiment were constitutively expressed in the liver, kidney, and testis (Fig. 1), whereas the mRNA abundance of certain isoforms showed tissue predominant expression such as GSTa1/2, GSTa3, and GSTt1. The expression of the same GST isoform varied among organs, such like GSTa1/2 and GSTa3 expression were highest in the liver, followed by the kidney and testis. However, the mRNA abundance of GSTm1, m2, m3, o1, o2, and MGST were highest in the liver, followed by the testis and kidney.

Variation of Transcription in Different GST Isoforms

The transcriptions of these GST isoforms also showed different temporal kinetics after exposure to MCs (Fig. 2). In the liver, the expressions of most GST isoforms were suppressed. The transcriptions of GSTa1/2, a3, a4, m2, m3, o1, t1, t2, and MGST were all suppressed and showed no recovery even at 24-h postinjection with MCs. The transcription of the GSTk isoform was relatively stable and showed no change from 1 to 24 h. The transcription of GSTm1 was induced at 2 h but suppressed at 12 and 24-h postinjection.

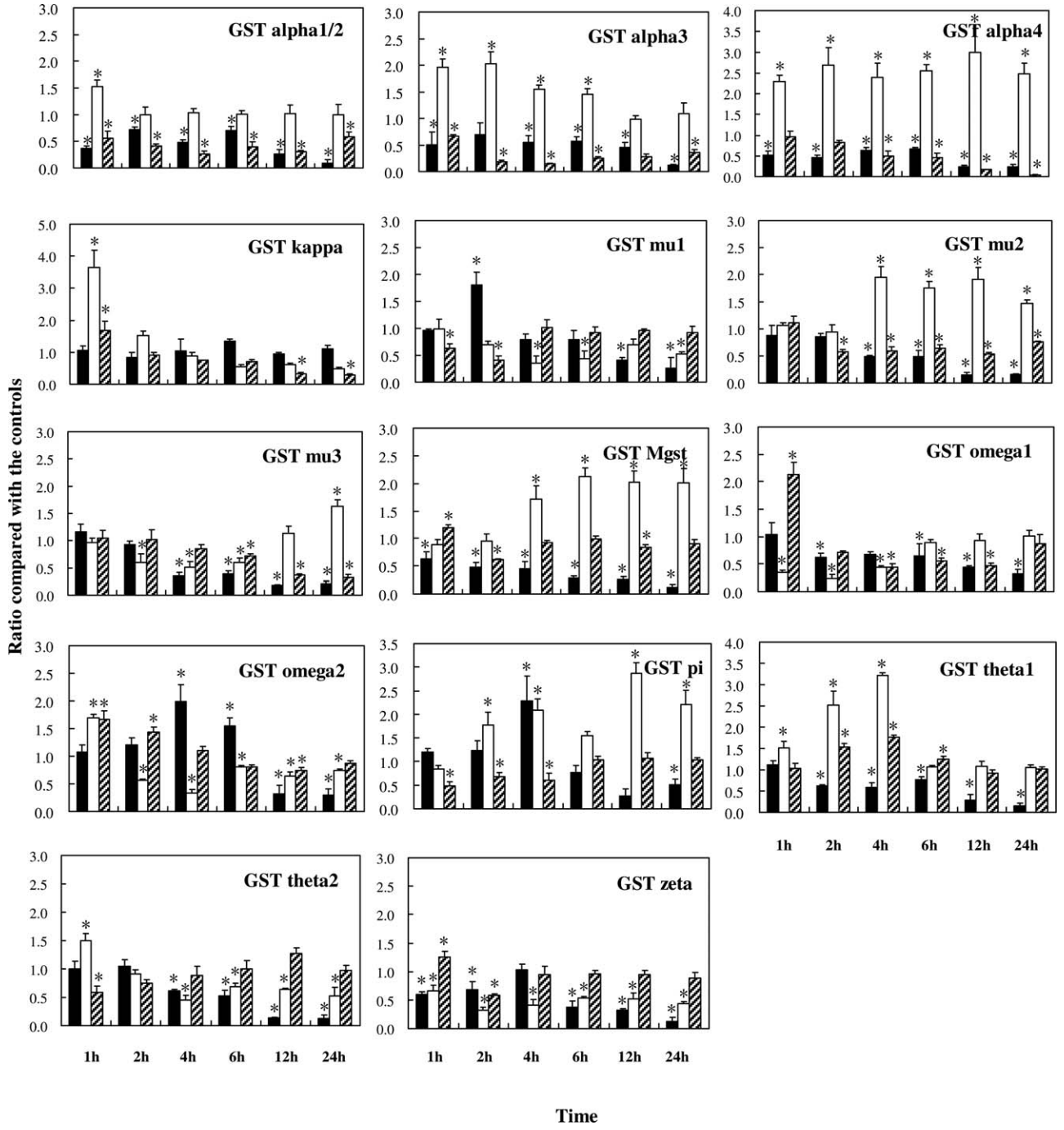

Fig. 2. The temporal changes of GSTs transcripts after microcystin stimulation compared with controls. ■ represents liver; □ represents kidney; ▨ represents testis (*indicates significant change at $P < 0.05$).

The transcription of GSTo2 increased at 4 and 6 h but was suppressed at the other time points.

In the kidney, the transcriptions of the GSTa1/2, a3, a4, k, o2, t1, and t2 were induced at 1-h postinjection with MCs. However, different transcription profiles among these isoforms were observed from 2 to 24-h postinjection. The expression of GSTa1/2 was induced at 2, 4, and 6 h but then reverted to the original level. The transcription of GSTa4 was induced at the following time and even showed no recovery tendency at 24-h postinjection. GSTo2 expression was inhibited at the following time. The transcription of GSTt1 was induced at 2 and 4 h and reverted to the original lever at 6-h postinjection. Whereas the transcription of GSTt2 was suppressed from 4 to 24 h and showed no

recovery even at 24-h postinjection. The transcriptions of the GSTm1, m2, m3, and MGST were relatively stable at 1-h postinjection. However, the expressions of GSTm1 and m3 were suppressed at 4 and 6 h, whereas the transcriptions of GSTm2 and MGST decreased from 4 to 24 h.

In the testis, the transcriptions of GSTa1/2, a3, a4, m2, and m3 were generally inhibited and showed no recovery tendency at 24-h postinjection. The transcriptions of GSTk, o1, o2, and MGST were all induced at 1 h but suppressed at 12-h postinjection with MCs. GSTm1 expression was suppressed at 1 and 2 h but then returned to the original level from 4-h postinjection. The transcription of GSTt1 decreased sharply at 1-h postinjection but then returned to the original level. The transcription of GSTt1 elevated from 2 to 6 h and then returned to the original level.

DISCUSSION

GSTs enzymes are the most extensively studied conjugation enzymes that are important in cellular protection against oxidative stress and toxic foreign substances. They detoxify a variety of internal and external toxicants, including MCs (Pflugmacher et al., 1998). Profiling metabolic enzymes, such as GST(s), within tissues allows for better predictions of potential sites of toxicity and metabolism in response to exposure to particular environmental pollutants (Mitchell et al., 1997).

Evaluation of mRNA levels is an established method to measure xenobiotic induction, and studies show that GST mRNA levels correlate closely with GST protein and enzyme activity levels (Buetler et al., 1995). In this study, we demonstrated that the phase II detoxification systems were altered by MC administration with changed transcript level of GST isoenzymes, suggesting different roles for GST isoenzymes in the detoxification of MCs-induced toxicity. It is well known that once absorbed into the blood, MC can be transported via the bloodstream rapidly and distributed to various organs, and the toxin is distributed especially in blood-irrigated organs like liver, intestine, kidney, and lung (Cazenave et al., 2005). MCs are potent hepatotoxins (Dawson, 1998) and tumor promoter by inhibiting protein phosphatase type 1 and 2A (Nishiwaki-Matsushima et al., 1991). On the other hand, it has been proven that MC-LR promotes renal alterations and affects renal physiology (Nobre et al., 2003; Moreno et al., 2003). Recently, Chen and Xie (2005) found that MCs could be effectively accumulated in gonads, which thus were regarded as the second target organs of MCs. Thus, it could be assumed that MCs affected the functions of the detoxification system of these organs.

In this study, distinct tissue-related differences in the expression of individual GST subunits were observed in the liver, kidney, and gonads postinjection with MCs. In the liver, the transcriptions of most GSTs (GSTa1/2, a3, a4, m2, m3, o1, t1, t2, and MGST) were all suppressed and showed no recovery even at 24-h postinjection with MCs. While in kidney, GSTa3, a4, m2, pi, t1, and MGST were all generally induced after MCs injection. In testis, the transcription of GSTa1/2, a3, a4, m2, and m3 were generally inhibited and showed no recovery tendency at 24-h postinjection. The transcriptions of GST isoforms in this study varied in different ways within an organ and among organs of rat exposed to MCs. The possible explanations for these variations could be attributed to (1) the MCs targets the liver, kidney, and testis, and its toxicity depends on the uptake and removal rates in these tissues (Ito et al., 2002). The accumulation of MCs in the liver and testis (Wang et al. 2008) may lead to the inhibition of most GST isoforms expression in these two tissues. This also implicates an increased health risk to Wistar rat. The induction of GSTs expression may release the pressure of kidney; and (2) the difference in the presence of xenobiotic-response and antioxidant response elements (AREs) between GST isoforms may determine the class-specific GST expression. Differential induction of phase II enzymes by the MCs may also be mediated with ARE in the genes through metabolic activation and an increase in oxidative stress. It seems that the GST gene expression is mediated by oxidative stress derived from a variety of chemicals. The rat GST alpha and mu gene are transcriptionally regulated by xenobiotics including phenolic antioxidants through activation of ARE-binding proteins (Pearson et al., 1988; Itoh et al., 1997). It should be appreciated that ARE is not the only transcription factor involved in regulating GST. The notion that Nrf2 (NF-E2 p45-related factor 2) mediates basal expression of GST by endogenous thiolactive endobiotics is supported by the fact in mice nulled for this transcription factor the normal homeostatic levels of many class Alpha, Mu, and Pi transferases are reduced (Hayes et al., 2000). That accumulation of MCs in different organs might influence these transcription factors (Itoh et al., 2004) and further influence the transcription of GST isoenzymes.

In our previous experiment, MCs also showed different effects on the transcription of GST isoforms in crucian carp (Li et al., 2008). In a microarray analysis, the transcription of GST alpha and mu in rats exposed to MC-LR at 50 μg kg^{-1} was elevated at 3 h and mGST1 decreased at 6 h, while pi had no change at 3 and 6 h post injection (Bulera et al., 2001). In another microarray analysis, when mice were exposed to MC-LR at 75% LD_{50}, transcription of the GST pi was elevated at 8h, and GST mu (mu2 and mu5 isoform) increased during 8–24 h (less than twofold compared with controls) (Gehringer et al., 2004). Such different results may be due to the difference in test animals, toxin level, exposure route, and sampling interval.

The present study examined the diverse expression of various GST isoforms in three organs of rat exposed to MCs. The toxicity of MCs depends on the balance between accumulation and metabolism (Ito et al., 2002). Meanwhile,

GSTs play important roles in the detoxification of MCs (Pflugmacher et al., 1998). It may be assumed that if the transcription of GSTs is inhibited, the rats are not able to eliminate MCs effectively. The variations of GSTs might affect capability of MC metabolization and subsequent cellular damages, physiological alterations, behavioral changes, and other effects (Kotak et al., 1996; Baganz et al., 1998, 2004; Fischer and Dietrich, 2000; Malbrouck et al., 2003). It is reasonable to assume that the suppression of many GST isoforms in the liver and testis might be a key mode of MC toxicity.

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