Rapid conversion and reversible conjugation of glutathione detoxification of microcystins in bighead carp (Aristichthys nobilis)

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The glutathione and cysteine conjugates of microcystin (MC-GSH and MC-Cys, respectively) are two important metabolites in the detoxification of microcystins (MCs). Although studies have quantitated both conjugates, the reason why the amounts of MC-GSH are much lower than those of MC-Cys in various animal organs remains unknown. In this study, MC-RR-GSH and MC-RR-Cys were respectively i.p. injected into the cyanobacteria-eating bighead carp (Aristichthys nobilis), to explore the biotransformation and detoxification mechanisms of the two conjugates. The contents of MC-RR, MC-RR-GSH, MC-RR-Cys and MC-RR-N-acetyl-cysteine (MC-RR-Nac, the acetylation product of MC-RR-Cys) in the liver, kidney, intestine and blood of bighead carp in both groups were quantified via liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). In the MC-RR-GSH-treated group, the MC-RR-Cys content in the kidney increased 96.7-fold from 0.25 to 0.5 h post-injection, demonstrating that MC-RR-GSH acts as a highly reactive intermediate and is rapidly converted to MC-RR-Cys. The presence of MC-RR in both MC-RR-GSH- and MC-RR-Cys-treated groups indicates, for the first time, that MC conjugation with the thiol of GSH/Cys is a reversible process in vivo. Total MC-RR concentrations dissociated from MC-RR-Cys were lower than those from MC-RR-GSH, suggesting that MC-RR-Cys is more capable of detoxifying MC-RR. MC-RR-Cys was the most effectively excreted form in both the kidney and intestine, as the ratios of MC-RR-Cys to MC-RR reached as high as 15.2, 2.9 in the MC-RR-GSH-treated group and 63.4, 19.1 in the MC-RR-Cys-treated group. Whereas MC-RR-Nac could not be found in all of the samples of the present study. Our results indicate that MC-RR-GSH was rapidly converted to MC-RR-Cys and then excreted, and that both glutathione and cysteine conjugates could release MC-RR. This study quantitatively proves the importance of the GSH detoxification pathway and furthers our understanding of the biochemical mechanism by which bighead carp are resistant to toxic cyanobacteria.

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1. Introduction

Microcystins (MCs) are a family of cyanotoxins that are widespread in eutrophic freshwaters around the world (Hilborn et al., 2007; Svrek and Smith, 2004). More than 80 structural analogs of MCs have been identified, with microcystin-RR (MC-RR) as one of the dominant variants in cyanobacterial blooms found in the lakes of China (Chen et al., 2006; Xie et al., 2005). MCs primarily act as hepatotoxins, leading to severe liver damage through inhibition of the serine/threonine protein phosphatases 1 and 2A. Consequently, exposure to MCs poses a health risk to wild and domestic animals, as well as to humans (Carmichael et al., 2001; Carmichael and Li, 2006; Li et al., 2012; Malbrunot and Kestemont, 2006; Zhang et al., 2010). In 1996, acute exposure to MCs led to the death of 76 patients during renal dialysis treatment in Caruru, Brazil (Azevedo et al., 2002). Recently, hepatotoxic microcystins were identified in the serum (average 0.228 ng MC-LR eq mL⁻¹) of a chronically exposed human population together with indications of hepatocellular damage (Chen et al., 2009).

Previous studies have shown that the GSH pathway plays an important role in the detoxification of MCs in various animals. The glutathione and cysteine conjugates were first identified as two metabolites of MCs in vivo in the liver of mouse and rat using the Frit–FAB LC/MS method (Kondo et al., 1992, 1996). Subsequently, the MC-LR glutathione conjugate has been demonstrated to form via glutathione-S-transferase (GST) extracted from various aquatic organisms. Thus, glutathione conjugation was suggested to be the first step in the detoxification of MCs, followed by degradation to the cysteine conjugate (Pluggmacher et al., 1998, 2001). As for MC-RR-Nac, the acetylation product of MC-RR-Cys, there has been no published study to quantitatively determine it in animal tissues. Until recently, an analytical method was established to

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simultaneously quantify MC-LR and its glutathione conjugate (MC-LR-GSH) in fish tissues (Dai et al., 2008). Then, Wu et al. (2010) also developed a novel method for identification and quantification MC-RR and its GSH pathway metabolites (MC-RR-GSH and MC-RR-Cys) in bighead carp by LC-ESI-MS. The establishment of these methods made it possible to study in depth the GSH detoxification process of MCs in aquatic animals.

Recently, several investigations have used the analytical methods described above to quantitatively study MCs and the corresponding metabolites in both field and laboratory conditions (He et al., 2012; Zhang et al., 2009, 2012). Because GSH conjugates of MC-LR were almost undetectable, whereas cysteine conjugates were dominant in wild aquatic animals, Zhang et al. (2012) doubted the importance of GSH conjugation with MCs and suggested that Cys conjugation might play the more important role in MCs detoxification. Extremely low concentrations of MC-LR/RR-GSH and high concentrations of MC-LR/RR-Cys were also observed in tissues from bighead carp in the laboratory. From this information, He et al. (2012) raised the hypothesis that MC-GSH might be rapidly changed to MC-Cys. However, no direct evidence has been reported so far to explain why the content of MC-GSH was much lower than that of MC-Cys in aquatic animals exposed to toxic cyanobacteria or purified MCs. Hence, determining the biotransformation and detoxification mechanisms of these two conjugates is essential for better understanding the roles of these conjugates in the detoxification of MCs in aquatic animals.

In the present study, equimolar doses of synthetic MC-RR-GSH and MC-RR-Cys were i.p. injected into bighead carp. We aimed to directly trace the toxicokinetics of these conjugates in vivo and to further identify metabolic characteristics of the GSH pathway. These results provide novel insights into the detoxification mechanisms of the GSH pathway. Furthermore, the results and research method hold promise for further studies comparing the metabolic detoxification mechanisms between aquatic invertebrates and mammals.

2. Materials and methods

2.1. Materials

MC-R was extracted and purified from Microcystis aeruginosa blooms collected from Lake Dianchi, China, by the method described in Ramanan et al. (2000) and Dai et al. (2008). The purity of MC-R was over 95%, as determined by HPLC (LC-20A, Shimadze, Kyoto, Japan). The identity of MC-R was confirmed using LC–MS (Thermo Electron, Waltham, MA, USA). L-Glutathione, L-cysteine and N-acetyl-L-cysteine were purchased from Acros Organics (Geel, Belgium), and the purity of the three was greater than 98%. MC-RR-GSH and MC-RR-Cys were formed using an improved method described by Wu et al. (2010). The purity levels of the two conjugates were over 95%, and the impure percentage (+5%) was not the parent MC-R but instead an unknown compound, as confirmed by HPLC and LC–MS (Fig S1). MC-RR-Nac was prepared by modification of the method of Dai (2008). Briefly, MC-RR-Nac (>95%) reacted with N-acetyl-L-cysteine in 5% potassium carbonate aqueous solution while stirring for 2 h at room temperature. The reaction mixture was first applied to an ODS C18 cartridge (2 g, Waters, Milford, MA, USA) and then was purified further by a semi-pre-LC (Waters 600, USA). The purity levels of MC-RR-Nac was over 95%, as confirmed by HPLC, and the chemical characterization of MC-RR-Nac was performed by (+)-ESI-MS/MS (Fig S2 and Table S1). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific International, Inc. (Fairfield, OH, USA). Ultrapure water was collected from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used were of analytical reagent grade.

2.2. Animal experiment and sample collection

Healthy bighead carp, Aristichthys nobilis, (weight 118 ± 9 g) were purchased from a local fish hatchery in WuHan, China. Fish were acclimatized in a 100-L aquaria containing dechlorinated tap water for 1 week before experimentation, and were fed pellets (1% of body weight per day) in a controlled environment (water temperature, 20 ± 1 °C; dissolved oxygen, 7.4 mg L⁻¹; pH, 8.4; photoperiod, 12 h light/dark). Upon initiating the experiment, the fish were divided into three groups at random, with 42 animals in each group. In two groups, fish were administered a single i.p. injection of approximately 0.5 mL suspension of the extracted solution of MC-RR-GSH or MC-RR-Cys. The dosages injected of both MC-RR-GSH and MC-RR-Cys were 0.55 μmol kg⁻¹ body weight (bw), maintaining an equimolar dose of MC-RR from our previous experiment (He et al., 2012). The control group was injected with the same volume of vehicle, which was 0.9% (w/v) saline solution. Considering that MC-RR-GSH might be rapidly converted to MC-RR-Cys, intensive sampling points were set during the first hour (0.25, 0.5, 0.75 and 1 h), followed by 2, 6 and 24 h, giving a total of seven time points in each group. At each time point, six randomly selected fish in each treatment group were sacrificed, and the liver, kidney, intestine (not including contents) and blood were rapidly collected and frozen at −80°C.

2.3. Sample preparation and analysis of MC-RR-GSH, MC-RR-Cys, MC-RR and MC-RR-Nac

The above tissue samples were freeze-dried by an Alpha 2–4 Freeze Dryer (Martin Christ, Osterode, Germany). Extraction and analysis of these lyophilized samples were performed following the methods described in Wu et al. (2010). Briefly, 0.2 g of freeze-dried sample was extracted three times in a 5 mL solution of 0.01 M EDTA-Na₂, 5% acetic acid by sonication for 3 min (30% amplitude, 60 W, 20 kHz, Branson Digital Sonifier, Danbury, CT, USA) at 0°C and then centrifuged at 12,000 r/min at 4°C (BR4, Jouan, Winchester, VA, France). Subsequently, the supernatant was applied to an Oasis MCX cartridge (3 cm³/60 mg, Waters, MA, USA), which was conditioned with 3 mL 100% MeOH and equilibrated with distilled water. The column was loaded with sample, washed with 3 mL 2% aqueous formic acid and 90% MeOH in sequence and then eluted with 10 mL of 15% aqueous ammonia in methanol. The eluent was dried by evaporation and redissolved in 100 μL of the mobile phase. Qualitative and quantitative analyses of MC-RR-GSH, MC-RR-Cys, MC-RR and MC-RR-Nac were performed by LC-ESI-MS. The parameters of this Finnigan LC–MS system were set up according to the previously described method (Wu et al., 2010). The limit of detection (LOD) of MC-RR, MC-RR-GSH, MC-RR-Cys and MC-RR-Nac was 0.005 μg g⁻¹ DW.

2.4. Statistics

All results were expressed as mean ± S.D. (standard deviation). Datasets did not allow for verification of normal distribution (according to the Shapiro–Wilk test), so non-parametric statistical analysis of SPSS for Windows (Ver. 13.0, Chicago, IL, USA) was used for the analysis of variance. Significant differences were determined at p < 0.05.

3. Results


Representative characterization of ESI LC/MS² analysis of MC-RR-GSH and MC-RR-Cys, in the kidney of bighead carp at 0.25 h
after MC-RR-GSH treatment as an example, is shown in Fig. 1. The relative retention times of MC-RR-GSH and MC-RR-Cys were 9.87 and 10.01 min, respectively. The ions present at m/z 609.05 and 537.03 were the product ions of MC-RR-GSH, with the corresponding precursor ion present at m/z 673.7. Similarly, the peak at m/z 580.2 was confirmed to be the precursor ion of MC-RR-Cys, with the corresponding product ions at m/z 513.46 and 520.11. Unexpectedly, a new peak appeared at a retention time of 10.72 min. Based on the presence of abundant fragment ions at m/z 453.02 and 887.43, derived from the precursor ion at m/z 520, we confirmed this peak to be MC-RR. Therefore, MC-RR as one metabolite was quantitatively detected in all of the samples.

However, MC-RR-Nac, of which the precursor ion was at m/z 601.61 and product ions were at m/z 534.43 and 537.06 (Fig. S2), could not be found in the liver, kidney, intestine and blood of bighead carp in both the MC-RR-GSH- and MC-RR-Cys-treated groups. In the control group, no MC-RR-GSH, MC-RR-Cys, MC-RR or MC-RR-Nac was detected during the experiment.

### 3.2. In vivo metabolism of MC-RR-GSH

Osmosis of MC-RR-GSH from the peritoneal cavity into the blood occurred rapidly after MC-RR-GSH treatment, as the MC-RR-GSH content reached as high as 3.998 ± 0.575 μg.g⁻¹ DW at 0.25 h post-injection (Fig. 2A). In the liver, the MC-RR-GSH content ranged from 0.141 ± 0.044 (0.25 h post-injection) to 0.009 ± 0.001 μg.g⁻¹ DW (24 h post-injection), declining rapidly during the experiment. The rapid, time-dependent decrease of MC-RR-GSH to the LOD was also observed during the experiment in the kidney and intestine. Statistical analyses showed that the levels of MC-RR-GSH were significantly higher in the kidney than in the liver (p<0.05).

In the blood, the MC-RR-Cys content increased gradually from 0.109 ± 0.000 (0.25 h post-injection) to 2.933 ± 0.284 μg.g⁻¹ DW (24 h post-injection) (Fig. 2A). A similar trend was observed in the liver (below LOD to 0.067 ± 0.006 μg.g⁻¹ DW). In the intestine, MC-RR-Cys levels ranged from 0.010 ± 0.001 to 0.070 ± 0.001 μg.g⁻¹ DW. In the kidney, however, the MC-RR-Cys levels sharply increased at 0.5 h, reaching a maximum
value of $4.638 \pm 0.890 \mu g g^{-1}$ DW, indicating a 96.7-fold increase within 0.25 h. The observed MC-RR-Cys contents were higher in the kidney than in both the liver and intestine ($p < 0.05$).

During the study period, MC-RR was consistently detected in all tissues (Fig. 2A). MC-RR was most abundant in the blood, with levels ranging from $1.672 \pm 0.476$ (0.25 h post-injection) to $0.945 \pm 0.172 \mu g g^{-1}$ DW (24 h post-injection). In the kidney, MC-RR exhibited almost the same trend as MC-RR-GSH. However, much lower levels of MC-RR were observed in both the liver, excluding the 0.25 h post-injection time point ($0.065 \pm 0.039 \mu g g^{-1}$ DW), and the intestine, excluding the 24 h post-injection time point ($0.215 \pm 0.000 \mu g g^{-1}$ DW).

3.3. *In vivo metabolism of MC-RR-Cys*

MC-RR-Cys accumulated abundantly in all analyzed organs after i.p. injection (Fig. 2B). In the intestine, the peak for MC-RR-Cys was observed at 0.25 h post-injection, representing the highest concentration observed in all of the tissues ($3.254 \pm 0.840 \mu g g^{-1}$ DW). The MC-RR-Cys levels in both the intestine and liver generally decreased in a time-dependent manner. However, the MC-RR-Cys content in the kidney was relatively stable until 2 h post-injection, maintaining levels evidently lower than those found in the intestine ($p < 0.05$). Then, the MC-RR-Cys levels increased to a peak ($1.839 \pm 0.029 \mu g g^{-1}$ DW) in a near-linear pattern until 24 h post-injection. In the blood, the MC-RR-Cys levels stayed relatively stable, ranging from $1.685 \pm 0.172$ to $2.705 \pm 0.544 \mu g g^{-1}$ DW.
Similar to the MC-RR-GSH-treated group, MC-RR was detected in all analyzed tissues after MC-RR-Cys treatment (Fig. 2B). The levels of MC-RR were much higher in the intestine than in the liver or kidney ($p < 0.05$), with a maximum concentration of $0.171 \pm 0.085 \, \mu g \, g^{-1} \, DW$, as compared with levels just slightly higher than the LOD in the liver and kidney. In the liver, MC-RR levels decreased from $0.027 \pm 0.002$ at $0.25 \, h$ post-injection to $0.009 \pm 0.000 \, \mu g \, g^{-1} \, DW$ at $6 \, h$ post-injection as the experiment progressed. In the kidney, MC-RR levels remained stable during the experiment, ranging from $0.013 \pm 0.002$ to $0.029 \pm 0.003 \, \mu g \, g^{-1} \, DW$. In the blood, the levels of MC-RR ranged from $0.125 \pm 0.043$ to $0.220 \pm 0.115 \, \mu g \, g^{-1} \, DW$.

The MC-RR-GSH contents in the liver, kidney and intestine were occasionally detected, and in the blood MC-RR-GSH always appeared below the LOD throughout the experiment (Fig. 2B).

### 3.4. Relationships between MC-RR-GSH, MC-RR-Cys and MC-RR

In the MC-RR-GSH-treated group of bighead carp, the MC-RR-GSH conjugates were converted not only to the major metabolite MC-RR-Cys but also to free MC-RR. MC-RR-Cys and MC-RR were detected in all analyzed tissues, while much higher MC-RR-Cys levels were observed in the kidney, and the intestine except for $24 \, h$ post-injection. At $0.5 \, h$ post-injection in the kidney, the ratio of MC-RR-Cys to MC-RR reached as high as $15.2$, while the ratio of MC-RR-Cys to MC-RR-GSH was $10.6$. In the intestine, the ratio of MC-RR-Cys to MC-RR was as high as $2.9$ at $2 \, h$ post-injection and the ratio of MC-RR-Cys to MC-RR-GSH was $4.2$.

Some MC-RR-Cys can release the parent compound MC-RR after MC-RR-Cys treatment in vivo. However, the observed levels of MC-RR-Cys were much higher than MC-RR in all tissues during the $24 \, h$ experiment. The maximum level of MC-RR-Cys was detected at $0.25 \, h$ in the intestine and the ratio of MC-RR-Cys to MC-RR was $19.1$, whereas the ratio of MC-RR-Cys to MC-RR reached as high as $63.4$ at $24 \, h$ post-injection. The total concentration of MC-RR in bighead carp refers to the combined concentration found in the liver, kidney, intestine and blood, and the concentration–time profile is shown in Fig. 3. Comparison between groups showed that the total MC-RR content detected in the MC-RR-GSH-treated group was significantly higher than that in the MC-RR-Cys-treated group ($p < 0.05$). These data suggest that, in bighead carp, the amount of MC-RR dissociated from MC-RR-GSH should be greater than that from MC-RR-Cys.

Total concentrations of MC-RR-GSH, MC-RR-Cys and MC-RR in the blood, intestine and kidney of bighead carp after treatment with either MC-RR-GSH or MC-RR-Cys are shown in Fig. 4. In the blood, total concentrations in the MC-RR-Cys-treated group were significantly lower than those in the MC-RR-GSH-treated group ($p < 0.05$). In the intestine, total concentrations in the MC-RR-Cys-treated group were higher than those in the MC-RR-GSH-treated group ($p < 0.05$). No significant differences were found between the total concentrations in the kidney and liver (data not shown). These data suggest that intestinal excretion might be a major, important factor for the rapid elimination observed in the blood.
4. Discussion

MC-GSH and MC-Cys have been identified as two important in vivo metabolites of the GSH detoxification pathway in various organisms (Kondo et al., 1996; Pflugmacher et al., 1998, 2001). Aside from the GSH pathway, MC-Cys may also be degraded from conjugates of MCs that directly interacted with proteins (mainly PP-1 and 2A) containing Cys residues in animals exposed to MCs (Zhang et al., 2012). However, in the present study, direct exposure to MC-GSH ensured that any MC-Cys detected in vivo was derived from MC-GSH. In the MC-RR-GSH-treated group, the MC-RR-Cys content increased 96.7-fold from 0.25 to 0.5 h post-injection, and the ratio of MC-RR-Cys to MC-RR-GSH at 0.5 h post-injection reached as high as 10.6. These results demonstrate that MC-RR-Cys could act as a reactive intermediate and could be rapidly converted into MC-RR-Cys. Therefore, the toxicokinetics of MC-GSH obtained here explain the results of previous studies (He et al., 2012; Zhang et al., 2009, 2012) reporting that MC-GSH levels were extremely low compared with MC-Cys levels in aquatic animals. This rapid conversion rate also agrees with the rapid degradation of S-substituted glutathione derivatives (t_{1/2} ≈ 3.5 s), as examined by microperfusion studies in rat kidneys (Anders, 1980). This high reactivity facilitates the formation of downstream products and determines an important role for MC-GSH in the detoxification process of MCs in bighead carp.

In the MC-RR-GSH-treated group, the MC-RR-Cys content was mainly distributed in the kidney, and a sharp increase was observed between 0.25 and 0.5 h post-injection. This organ preference may be attributed to a number of factors. First, a number of carriers, such as members of the oatp/OATP superfamily, help to transport MC-RR-GSH to the kidney and/or help to transport MC-RR-Cys produced by other organs, such as the liver, into the kidney (Fischer et al., 2005; Hagenbuch and Meier, 2003; Lohr et al., 1998; Meier et al., 1997; Meier and Steiger, 2002). Additionally, the MC-RR-GSH arriving in the kidney was rapidly and effectively converted to MC-RR-Cys, as high gamma-glutamyltranspeptidase (GGT) and cysteinylglycine dipeptidase activities, which catalyze this conversion process, were found in the kidneys of mammals and fish (Elfarra and Anders, 1984; Hughey et al., 1978; Kera et al., 1999; Law et al., 2012). Previous studies observed significant induction of GGT genes in medaka larvae within 15 min after treatment with tert-butyl hydroquinone (tBHQ) in the rearing solution (Law et al., 2012). Based on these related research data together with the present results, we infer that the renal metabolism may play a major role in the processing of MC-RR-GSH into MC-RR-Cys in fish like bighead carp, as well as in the accumulation of MC-RR-Cys in the kidney for excretion. Moreover, the intestine was shown to be another location for the excretion of MC-RR-Cys, as a rapid and higher accumulation was observed in the early stage in the MC-RR-Cys-treated group. Consistent results have also been reported by Ito et al. (2002), showing that MC-LR-Cys was prominently detected in the kidney and intestine but not in the liver of mice, as observed after intratracheal administration using an immunostaining method. All of these results suggest that the cysteine conjugates of MCs mainly accumulate in excretive organs (kidney and intestine) and are the effectively excreted form in animals.

Traditionally, GSH conjugates are enzymatically cleaved down to cysteine conjugates, and then N-acetylated for form mercapturic acid conjugates which are ultimately excreted out of the body, for instance, through the urinary excretion (Fischer et al., 1985). However, MC-RR-Nac, could not be found in all the samples of the present study. The possible reasons for the absence of mercapturic acid conjugates are explained as follows. First, the content of MC-RR-Nac, at the terminal of the metabolic pathway, was very low, and complex endogenous compounds from matrices might bring great interference during LC–MS analysis of the trace MC-RR-Nac. Second, we focused on the rapid conversion of the MC-RR-GSH conjugate in bighead carp, and MC-RR-Nac may appear with the extension of the time rather than at the early stage (0–24 h, experimental period of the present study). Third, the absence of MC-RR-Nac in all samples of bighead carp may indicate a lack or low activity of N-acetyltransferase in this species.

Bighead carp, the freshwater phytoplanktivorous fish, are able to graze on toxic M. aeruginosa blooms in non-traditional biomanipulation (Xie and Liu, 2001). In our present study, the GSH pathway is quantitatively shown to be an important biochemical mechanism for bighead carp to resist toxic cyanobacteria. In bighead carp, MCs conjugate with GSH to form the MC-GSH conjugate, which is subsequently rapidly degraded to the cysteine conjugate and finally excreted from the kidney and intestine in the form of MC-Cys.

The presence of MC-RR in bighead carp exposed to MC-RR-GSH and MC-RR-Cys suggests that MC-RR can be dissociated from the MC-conjugates. MC conjugation with the thiol of GSH/Cys was, for the first time, found to be a reversible process in vivo. The thiol of GSH/Cys was confirmed to be added nucleophilically to the α,β-unsaturated carbonyl of the Mdha moiety in MCs (Kondo et al., 1992). This reversible behavior of MC-conjugates is in agreement with the theory proposed by Monsk et al. (1990) that the occurrence of Michael and “retro-Michael” reactions can be realistically expected for the classic substrate, α,β-unsaturated aldehydes or ketones. Two other classes of compounds, isocyanates and isothiocyanates, have also been shown to undergo a reversible GSH conjugation reaction (van Bladeren, 2000). It was reported that 9–15% of the benzyl and allyl isothiocyanate conjugates were converted to free isothiocyanates within 15 min when the equilibrium was established. Furthermore, as much as 95% of the α-naphthylisothiocyanate-GSH (ANIT-GSH) released free ANIT within 5 min after the conjugates were dissolved in buffer at natural pH in the absence of free GSH (Bailie and Kassahun, 1994). Moreover, the relatively rare compound dichloromethane also undergoes conjugative metabolism, resulting in the formation of formaldehyde and glutathione via mammalian GST or bacterial dehalogenase (Stourman et al., 2003; Wheeler et al., 2001a,b).

In the present study, although the proportion of free MC-RR released from MC-RR-GSH/Cys was unknown when equilibrium was reached, relatively high concentrations of MC-RR were indeed observed in various organs, especially in blood samples from both MC-RR-GSH- and MC-RR-Cys-treated groups. Compared with the MC-conjugates, the toxicity of the parent MCs is higher. For example, the LD_{50} of MC-LR is just 0.06 times that of MC-RR-GSH and 0.14 times that of MC-RR-Cys (Kondo et al., 1992). Hence, the MC-RR released from MC-RR-GSH/Cys in vivo, may play an important role in the appearance of toxicity in MC-RR-GSH/Cys-exposed bighead carp.

The detoxification of MCs via GSH is made more complicated due to the reversibility of thiol conjugation. Due to the release of MC from MC-conjugates, MC-GSH/Cys may also serve as forms of transport or storage in vivo. In this sense, conjugation of MCs with GSH might not fully achieve a conventional “detoxification” goal. In the present study, after MC-RR-GSH and MC-RR-Cys treatment, free MC-RR was mainly distributed in the blood, followed by the kidney and intestine, but not in liver (Fig. 3). Thus, some of the glutathione conjugates, which formed mainly in the liver by GST, released free MCs at sites distant from the liver via blood circulation or a different transport system. This behavior suggests the MC-GSH conjugates may mediate multiple organ toxicity beyond the well-known hepatotoxicity of MCs.

In the present study, total MC-RR concentrations in bighead carp released from the MC-RR-GSH/Cys-treated group were much lower than those from the MC-RR-GSH-treated group (Fig. 3), possibly due to different detoxification capacities. In other words, fewer parts of MC-RR were dissociated from MC-RR-Cys after MC-RR was conjugated with Cys, and MC-RR-Cys was more capable of
detoxifying MC-R in fish. Furthermore, rapid blood clearance was observed after MC-RR-Cys exposure because the total concentrations in blood were much lower in the MC-RR-Cys-treated group compared with those in the MC-RR-GSH-treated group (Fig. 3A). After further comparison of total concentrations in the liver, kidney and intestine of both groups, higher total concentrations in the intestine (Fig. 3B) were identified in the MC-RR-Cys-treated group, suggesting that intestinal excretion is important for rapid blood elimination. Additionally, the present results, together with a previous study (He et al., 2012), demonstrated that the kidney plays an important role in excretion. MC-RR-Cys conjugates, composing the dominant fraction of the total concentration, were rapidly eliminated from the systemic blood circulation through intestinal and renal excretion routes, thus ensuring rapid and effective detoxification. Finally, MC-Cys exhibited almost the same inhibitory activity as MC-GSH against protein phosphatases 1 and 2A under in vitro experimental conditions (Ito et al., 2002), indicating that the toxicity of MC-Cys, as one product of the GSH pathway, did not increase. Taken together, the biochemical characteristics of cysteine conjugates of MCs are crucial in supporting the detoxification process, further demonstrating the importance of the GSH pathway in detoxification of MCs.

5. Conclusions

(1) MC-RR-GSH can act as a highly reactive intermediate and is rapidly converted to MC-RR-Cys.

(2) The presence of free MC-RR demonstrates, for the first time, that MC conjugation with the thiol of GSH/Cys is a reversible process in bighead carp.

(3) The total MC-RR concentrations dissociated from MC-RR-Cys observed were lower than those from MC-RR-GSH, suggesting that MC-RR-Cys is more capable of detoxifying MC-RR.

(4) The GSH detoxification pathway was quantitatively proven to be an important biochemical mechanism for bighead carp to resist toxic cyanobacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.12.001.

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